Minimizing the risk of staphylococcal food poisoning

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Habilitationsschrift zur Erlangung der *Venia legendi* der
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vorgelegt von

Dr. med. vet. **Sophia Johler-Ilić**
von Kempten, Deutschland
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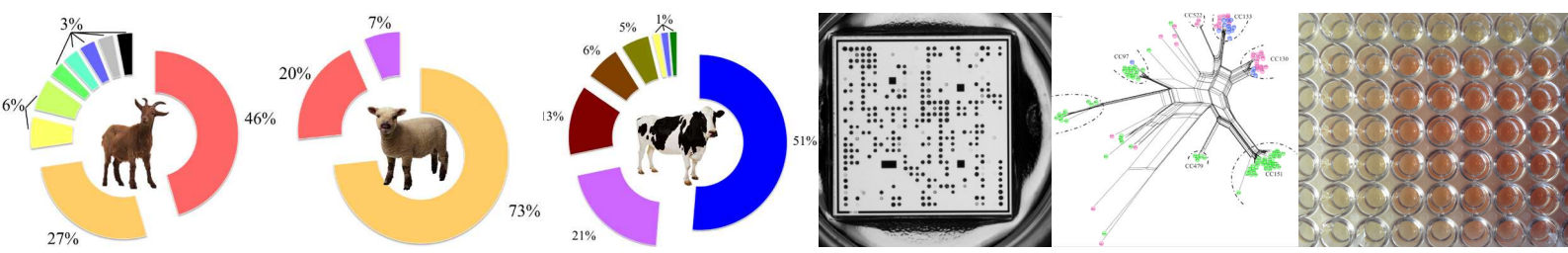

Cover Illustrations (from left to right):

- Clonal complex distribution in *S. aureus* from caprine, ovine, and bovine milk
- DNA microarray hybridization profile
- SplitsTree visualizing similarity of virulence and resistance gene profiles of *S. aureus* from different sources
- Nitrocefin assay used to determine seb promoter activity in transcriptional fusions under nitrite stress

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Dedicated to my doctoral students

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1 Abstract

Staphylococcal food poisoning is the most prevalent cause of food-borne intoxications worldwide. Consumption of enterotoxins preformed in food by *Staphylococcus (S.) aureus* causes violent vomiting and can be fatal in children and the elderly. When this habilitation thesis was devised, the most likely sources of staphylococcal food poisoning were still unclear, the expression and regulation of enterotoxins under stress conditions encountered during food production and preservation was poorly understood, and data on outbreaks and product-related studies were scarce. Therefore, the overarching objective of this habilitation thesis was to generate data that can be utilized to minimize the risk of staphylococcal food poisoning by i) identifying the genomic background and the most common sources of staphylococcal food poisoning, ii) examining enterotoxin expression and regulation under food-related stress conditions, and iii) investigating outbreaks and adapting food production parameters to inhibit *S. aureus* growth and enterotoxin formation. To reach these goals, a multifaceted approach was chosen. To obtain data on the genomic level, enabling source attribution and insights into the population structure, *S. aureus* linked to foodborne outbreaks and *S. aureus* strains from various sources were characterized by whole genome sequencing, DNA microarray profiling, and different further typing techniques. To investigate enterotoxin expression and its regulation under stress conditions, enterotoxin expression was quantified in different growth phases and under several food-related stress conditions in multiple wild type and isogenic regulatory knockout strains using a quantitative Real-Time approach and an ELISA assay. To yield data on staphylococcal outbreaks and to determine effective strategies to minimize the risk of staphylococcal food poisoning, several outbreak investigations were conducted and a cheese production process was optimized in order to inhibit *S. aureus* growth and enterotoxin production. The results of these studies contributed in various ways to the current body of knowledge on staphylococcal food poisoning. A novel pathogenicity island carrying *seb* in a staphylococcal food poisoning outbreak strain was described. In addition, we were able to show that food handlers colonized by or infected with *S. aureus* are the most common source of staphylococcal food poisoning outbreak strains. In contrast, *S. aureus* present on pig, poultry, and rabbit carcasses as well as in bovine, ovine, and caprine milk seem to play only a minor role in staphylococcal food poisoning outbreaks. With regard to enterotoxin expression and regulation under stress conditions encountered during food production and preservation, we were able to show that NaCl and glucose stress reduce enterotoxin D expression, whereas lactic acid stress had no significant effect. In contrast, nitrite stress induced enterotoxin D expression in some strains. These findings suggest the production of staphylococcal enterotoxins cannot be reliably predicted based exclusively on viable cell counts. Legislative guidelines need to be adjusted accordingly in order to assure consumer safety. As for the effect of regulatory elements, we were able to show that sigma factor B plays a major role in the

regulation of enterotoxin expression, whereas the role of the accessory gene regulator may have been overestimated in the past. Overall, strong strain-specific variation in both expression of enterotoxins under stress and the role of regulatory elements was observed, emphasizing the need for a multiple strain approach when investigating regulatory mechanisms and virulence factor expression in *S. aureus*. Using the findings of outbreak and product related studies, we were able to show that newly-described staphylococcal enterotoxins encoded by the *egc* cluster can cause outbreaks of staphylococcal food poisoning. In addition, we identified high-level contamination of raw milk and raw milk soft cheese with *S. aureus* of genotype B, originating from cow herds with high within-herd prevalence of mastitis, as a risk factor for staphylococcal food poisoning outbreaks. Our findings also indicate that incubation periods can depend of the age of the patient, with 2.5 h in children under 10 years of age, 3.5 h in older children and teenagers, and 7 h in adults. Finally, we successfully altered a barbecue cheese production process. In a model cheese production and a challenge assay, we were able to show that the new starter culture inhbits *S. aureus* growth and enterotoxin formation, while meeting sensory and technological requirements, thus minimizing the risk of staphylococcal food poisoning.

2 Introduction

Staphylococcus (S.) aureus is an organism of striking versatility. Comfortably persevering in inanimate sites, it is able to colonize and infect both humans and animals, with clinical implications ranging from harmless commensal states to life-threatening systemic infections and toxinoses (1). *S. aureus* is also the most prevalent pathogen implicated in food-borne intoxications worldwide. Strains can produce one or several staphylococcal enterotoxins, which lead to violent vomiting, diarrhea, and abdominal cramping upon ingestion.

Approximately 30% of the healthy adult population are persistent nasal carriers of *S. aureus,* with an additional 30% being classified as transient carriers (2–4). While nasal colonization is usually asymptomatic, it serves as a reservoir for the spread of the organism (2, 5, 6) and predisposes the carrier to post-surgical infections (5, 7). Prophylactic intranasal mupirocin application was reported to significantly reduce the rate of post-surgical infections in *S. aureus* carriers (6). However, decolonization is only temporary and the rise of mupirocin-resistant *S. aureus* is a cause for concern (8, 9).

S. aureus can also cause a wide range of infections and even though host tropism and adaptation have been described (10, 11), most *S. aureus* strains can readily be transmitted from humans to animals and vice versa (12). In humans, *S. aureus* is frequently isolated from cases of skin and wound infections and represents a leading cause of infective endocarditis and sepsis (13, 14). *S. aureus* can also colonize or infect companion animals as well as farm animals used for food production, including ruminants, pigs, rabbits, and poultry. In ruminants, *S. aureus* is a major cause of intramammary infections, leading to severe financial losses for the dairy industry (15, 16).

The rise of methicillin resistant *S. aureus* (MRSA) has become a major public health concern worldwide. Historically associated with hospitals, MRSA has emerged as a common cause of livestock- and community-associated infections (17). MRSA infections have been estimated to constitute the leading cause of death due to one single infectious agent in the United States (18).

The diversity of *S. aureus* pathomechanisms is largely based on the broad spectrum of exoproteins the organism produces. The vast majority of strains secretes various hemolysins, nucleases, lipases, proteases, leukocidins, collagenase, and hyaluronidase that enable *S. aureus* to convert host tissue into nutrients essential to bacterial growth (19). Many strains also produce additional secreted superantigenic exoproteins such as exfoliative toxins, toxic shock syndrome toxin-1, and enterotoxins (19).

Relevance, symptoms, and sources of Staphylococcal Food Poisoning

Staphylococcal Food Poisoning, the most prevalent foodborne intoxication worldwide, is caused by ingestion of staphylococcal enterotoxins (SEs) preformed by *S. aureus* in food (Figure 1). As clinical symptoms are often self-limiting, only few SFP patients are admitted to a hospital or consult a physician (20, 21). Therefore, individual cases of SFP are seldom reported and the organism is primarily isolated in large outbreaks.

A study of the USDA Economic Research Service estimated *S. aureus* to be responsible for 1.2-1.5 million cases of food poisoning per year, causing annual costs of $1.2 billion (22). With regard to morbidity and mortality, the Centers for Disease Control estimate that 240,000 cases per year occur in the US alone, leading to 1,000 hospitalizations and six deaths (23). While similar numbers are not available for Europe, EFSA listed 393 reported outbreaks for the EU and Switzerland in 2014 (24). As many minor staphylococcal food poisoning outbreaks are not being reported, the real incidence is estimated to be considerably higher (25).

Staphylococcal food poisoning is typically self-limiting, presenting with emesis following a short incubation period. Symptoms of acute gastroenteritis start 30 min to 8 h after ingestion of staphylococcal enterotoxins and spontaneously subside after 24 h (26). In a survey of clinical symptoms of 2,992 patients suffering from staphylococcal food poisoning, the key symptom described was vomiting (82% of the cases), often combined with nausea

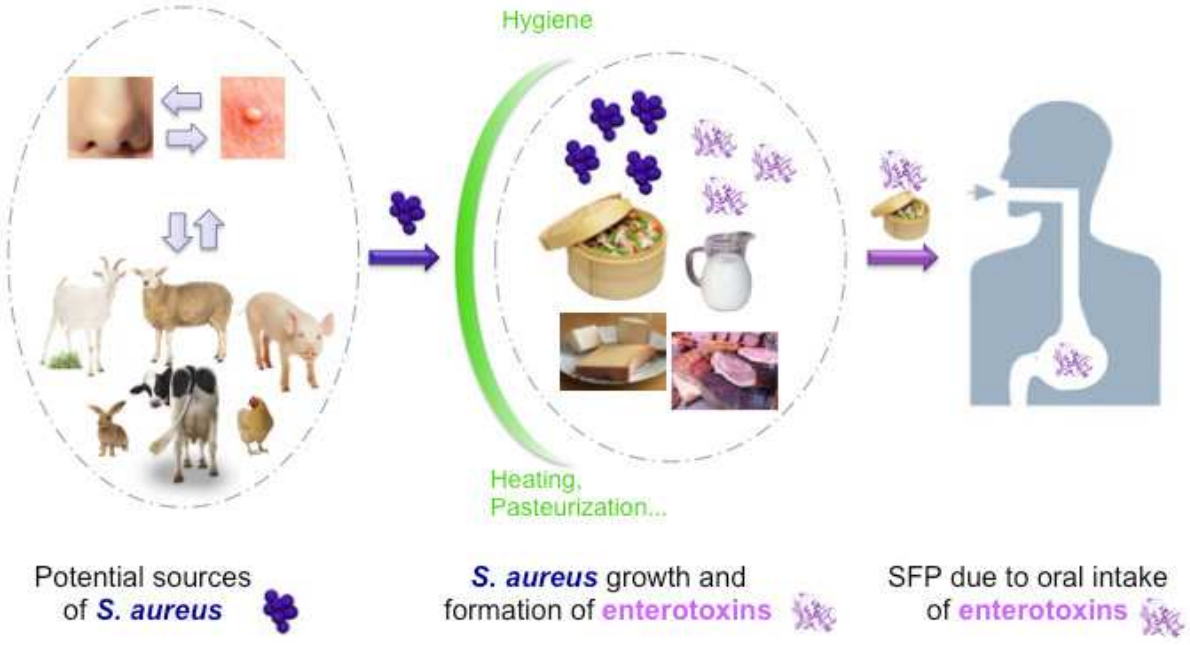


Figure 1: Staphylococcal food poisoning. Humans and various food-producing animals colonized or infected with *S. aureus* represent sources that can introduce enterotoxigenic *S. aureus* to the food matrix. During subsequent growth of *S. aureus* in food, enterotoxins can be formed. Oral intake of enterotoxins leads to staphylococcal food poisoning. In this process, the organism itself is not required for an emetic response. Even if *S. aureus* is inactivated during the food preparation process, the enterotoxins remain emetically active and can cause staphylococcal food poisoning.

(74%), diarrhea (68%), and abdominal pain (64%) (27). In rare cases, staphylococcal food poisoning can also result in fatal dehydration and electrolyte imbalances. Fatality rates range from 0.03% for the general public to 4.4% in sensitive populations such as children and the elderly (27). While contamination of food by food handlers was suggested to likely play an important role in staphylococcal food poisoning, the most common sources of staphylococcal food poisoning strains are still unclear and may also be influenced by the eating habits of specific countries (25, 26)

Staphylococcal enterotoxins

SE are water-soluble, short proteins of 194–245 amino acids that belong to the family of pyrogenic toxin superantigens and are often located on mobile genetic elements including plasmids and various *S. aureus* pathogenicity islands (SaPIs) (28–30).

SEs are designated following an alphabetical nomenclature system (19). The "classical" or "major" SEs (SEA, SEB, SEC, SED, and SEE) are traditionally differentiated from newly described SEs or SE-like superantigens. (31–33). The International Nomenclature Committee for Staphylococcal Superantigens (INCSS) proposed the following guidelines for the description of SEs (31–33): Before new superantigens or superantigen-like toxins can be described, the expression of the respective gene has to be verified and the protein must be characterized. Moreover, emetic activity of any new SE must be verified in a monkey feeding assay. Toxins that lack emetic activity in this assay or have not been tested yet, should be designated "staphylococcal enterotoxin-like superantigens".

When this habilitation thesis was devised, the role of newly described SEs in staphylococcal food poisoning and in infectious diseases was still controversially discussed. Over the course of the last years, interest in newly-described SEs rose, and a recently published study shows that *egc*-encoded newly described SEs result in local tissue effects essential for the establishment and progression of infective endocarditis (34).

All major SEs and to a lesser degree also most of the newly described SEs, including SEG and SEI, can elicit an emetic response in a monkey feeding assay (21, 35). However, it was shown that strains harboring *seg* and *sei* only produce very low levels of SEG and SEI (36) and only weak emetic activity was demonstrated for SEI (37). Thus, only for the major SEs and SEH (38–40), there is conclusive evidence demonstrating emetic activity in humans. Still, in recent years, there is a growing number of studies indicating that SEG and SEI may be responsible for cases of staphylococcal food poisoning in humans (41, 42).

Administering SEK-SEQ to cynomolgus monkeys at 100 µg/kg showed weak emetic activity and resulted in vomiting of two in six (SEK, SEN, SEQ), one in six (SEL), one in seven (SEM), and one in eight (SEO) animals, respectively (35). ED_{50} of major SEs in monkeys ranges between 0.9 and 20 µg/kg (43, 44). In rhesus monkeys, SEG and SEI provoked diarrhea or pronounced lethargy in all tested animals and emesis in four out of six

(80 µg/kg SEG) and one out of four (150 µg/kg SEI) animals (37). While the emetic activity of SEI in the rhesus monkey seems rather low, it provokes vomiting in the house musk shrew at an emetic activity comparable to SEA and far exceeding SEB, SEC, and SED (45).

Outbreak investigations

Data on staphylococcal food poisoning outbreaks is scarce and often incomplete. This is partly due to the fact that the intoxication is typically self-limiting within 24 hours, with only 10% of patients seeking medical attention (20, 21). In addition, SEs are heat-stable and display high tenacity in the face of stressors that reliably inactivate the organism itself. SEs also remain emetically active in the gastrointestinal tract, because they can resist most proteolytic enzymes, including pepsin and trypsin. Therefore, the organism that caused a staphylococcal food poisoning outbreak may not be retrievable from food or feces in the course of an outbreak investigation.

The role of newly-described enterotoxins in staphylococcal food poisoning outbreaks has been controversially discussed for years, although most of these toxins elicit an emetic response in the monkey feeding assay (21, 35). Emetic activity in humans was only demonstrated for the major enterotoxins SEA-SEE (19) and the newly-described enterotoxin SEH (38–40). The discussion is further complicated by the fact that in many outbreaks strains both major and newly described SE genes can be detected, but only classical enterotoxins can be identified in food and feces by commercially available immunological based methods. Therefore, even if only low levels of SEA-SEE will be detected in food or feces, the outbreak will often be attributed to one of the major enterotoxins.

SE production under stress conditions

There is some data available on enterotoxin expression under non-stress conditions (46–48). However, the effect of food-related stress on enterotoxin expression is unclear. Previous studies investigating the influence of stress on enterotoxin expression largely focused on enterotoxin B and relied on immunological methods (49–53). However, loss of serological recognition does not equal loss of biological/emetic activity (26, 54).

While growth of *S. aureus* is repressed by competing bacteria in most food matrices, the organism exhibits a crucial growth advantage under stress conditions including pH and salt stress (55, 56). Currently, legislative guidelines try to minimize the threat of intoxications by limiting the maximum amount of *S. aureus* in food, neglecting the imminent threat arising from unforeseeable enterotoxin production under stress conditions. *S. aureus* is able to grow at a_w values < 0.90 (2.6 M NaCl), rendering it one of the most osmotolerant food-borne pathogens (56). Data on the effect of stressors on enterotoxin formation is urgently needed to minimize enterotoxin formation during food production and preservation.

While two older studies report that salt stress

affects enterotoxin formation (50, 51), more recent studies suggest that SEA formation increases under mild lactic and acetic acid stress (57, 58). A French research group investigated *sed* expression in cheese (47), but did not elucidate the role of regulatory elements and specific stress factors. Schelin and coworkers started to systematically investigate enterotoxin production in the food matrix and under stress conditions including first experiments for SED (58–62). However, no experiments allowing insights into the role of regulatory elements were included.

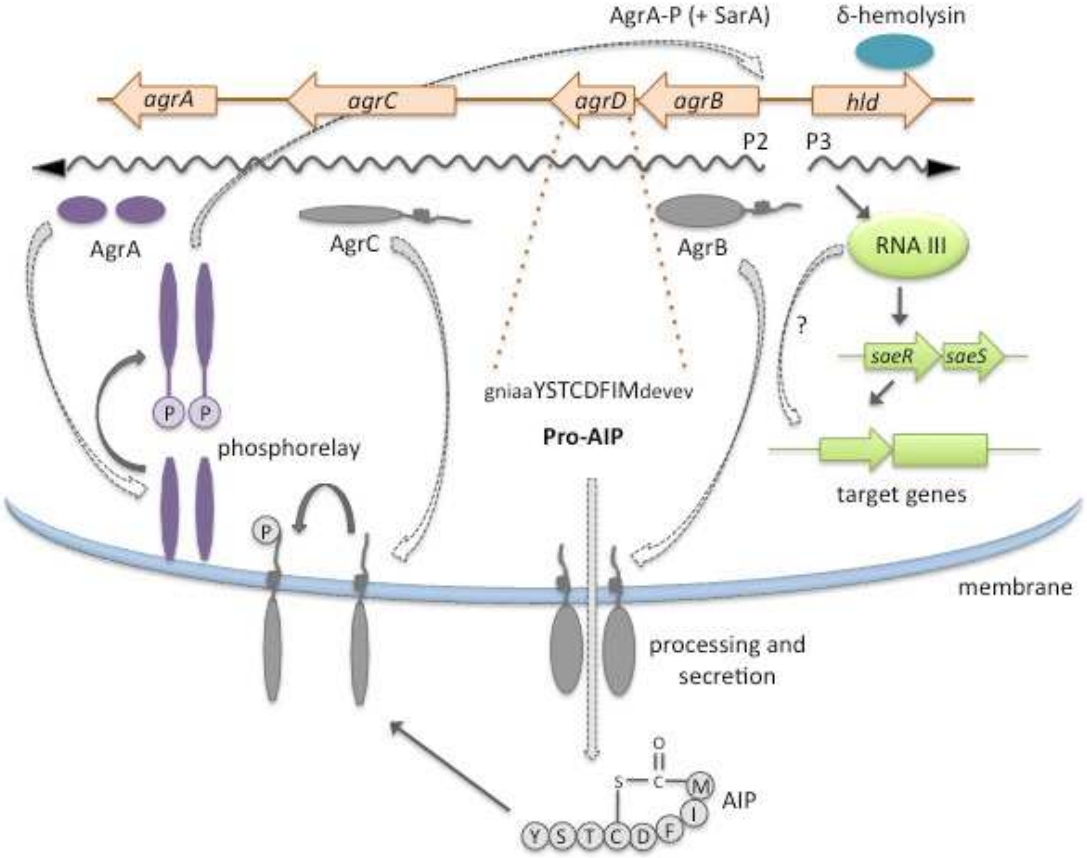
Regulation of enterotoxin expression

A complex network of interacting regulators influences the expression of enterotoxin genes. The accessory gene regulator (Agr), the staphylococcal accessory regulator (SarA), and the alternative sigma factor B (σ^B) have been shown to be involved in regulation of *seb*, *sec*, and *sed* expression.

Data is however limited, especially considering enterotoxin regulation under stress conditions. In addition, data from previous studies investigating regulatory mechanisms controlling the expression of major SEs need to be treated with caution, as most experiments were performed using strain NCTC8325. This strain and its derivatives exhibit a 11-base deletion in *rsbU*, which encodes a phosphatase activating σ^B (63). Moreover, there are important differences in regulatory mechanisms among *S. aureus* strains (68), suggesting that data generated using only one strain cannot be extrapolated to *S aureus* in general.

The quorum sensing dependent ***agr*** system is involved in the response of *S. aureus* to environmental stimuli. It is a two-component regulatory system, which responds to an autoinducer peptide (69). Expression of this global regulator coincides temporarily with maximum expression of most SE, excluding SEA and SED, which are expressed earlier (27). In general, activation of *agr* was shown to inhibit expression of genes encoding certain cell-wall associated proteins and increasing expression of genes encoding exoproteins during the post-exponential phase of growth (70, 71). The *agr* system regulates *seb*, *sec*, and *sed* on the level of transcription. In mRNA mutants, steady-state levels were reduced 4-fold for *seb*, 5.5-fold for *sed*, and 2 to 3-fold for *sec* (72). Reductions in enterotoxin protein synthesis were even more pronounced, showing 16 to 32-fold reductions in SEC and 5-fold reductions in SED, as evidenced by Western blot analysis (69). The mechanism of action of the *agr* locus is depicted in Figure 2. Transcription can be initiated by three promoters (P1, P2, P3). P1 transcribes *agr*. The P2 transcriptional unit RNAII encodes the proteins AgrA, AgrB, AgrC and AgrD. These proteins form a quorum sensing system and are required for the transcription of P2 and the activation of P3 (27, 70). The partially translated P3 transcript, RNAIII, is the effector of the *agr* locus (70). The auto-inducing peptide AIP represents the activating molecule for the *agr* system. The AgrD-encoded propeptide, derived from AgrD-residues 46-53, was suggested to be processed and secreted by AgrB (68). AIP binds to an extracellular loop of the transmembrane-histidin-kinase AgrC, a membrane bound receptor recognizing

Figure 2: The *agr* locus in *S. aureus* modified after Doyle and Novick (68, 97). The auto-inducing peptide AIP represents the activating molecule for the *agr* system. The AgrD-encoded propeptide derived from AgrD-residues 46-53, was suggested to be processed and secreted by AgrB. AIP binds to AgrC, a membrane-bound receptor recognizing increased levels of AIP in the environment. The following autophosphorylation enables phosphorylation of the response regulator AgrA. In combination with SarA, this leads to the activation of the *agr* promoters P2 and P3 and subsequent expression of RNAII and RNAIII.



increased levels of AIP in the environment. The following autophosphorylation enables phosphorylation of the response regulator AgrA. In combination with SarA this leads to activation of the *agr* promoters P2 and P3 and expression of RNAII and RNAIII (27, 68, 73).

The ***sarA*** locus binds several promoters, including *agr, hla, spa* and *fnbA*. It interacts with an intergenic region between *agr* promoters P2 and P3, which upregulates expression of RNAIII, thus influencing the expression of *agr* regulated genes. Moreover, transcriptional gene fusion has shown that *sarA* upregulates the toxic shock syndrome toxin gene and *seb* (27, 69).

Alternative sigma factors play an important role in the response to environmental stimuli. *S. aureus* harbors only one alternative **sigma factor** – σ^B, which has an opposite effect than Agr on the expression of several exoproteins and can be activated by environmental stress and energy depletion (1). It was reported to influence the transcription of over 250 genes, including upregulation of the expression of adhesins and downregulation of the expression of many exoproteins (74). The expression of *seb* was suggested to be repressed by σ^B in an *agr*-independent mechanism. Therefore, induction of σ^B by environmental stress leads to decreased expression of *seb* (27, 74).

S. aureus typing

The most widely used methods for *S. aureus* subtyping are *spa* typing, pulsed field gel electrophoresis (PFGE), and multilocus sequence typing (MLST).

spa typing is based on variable-number tandem repeats of the *spa* gene encoding protein A. Based on nucleotide sequence variations in the polymorphic X region of *spa*, isolates are assigned to *spa* types, allowing for epidemiological and phylogenetic conclusions. While this tool is rapid, robust, and highly discriminatory, it may fail to identify new lineages as a result of inherent homoplasy and differing evolutionary rates of *spa* alleles (75).

Many laboratories still consider PFGE using *SmaI* restriction the gold standard in *S. aureus* fingerprinting. However, PFGE is time-consuming and exhibits severe performance discrepancies when used in inter-laboratory comparisons (76). In addition, due to methylation, *S. aureus* of ST398, including livestock-associated MRSA, are refractory to *SmaI* restriction and therefore non-typable using this method.

The assignment of sequence types and clonal complexes by MLST is based on determining the sequences of seven housekeeping genes of *S. aureus*. The method is robust, but offers only moderate discriminatory power at comparatively high cost (77, 78).

3 Objectives

The objective of the studies combined in this habilitation thesis was to contribute significantly to the understanding of staphylococcal food poisoning, with the overarching goal of utilizing this knowledge to minimize the risk of staphylococcal food poisoning. To achieve a comprehensive picture, a multifaceted approach was chosen, targeting SFP on the genomic level (**4.1**), on the level of enterotoxin expression and regulation (**4.2**), and on the level of outbreak investigations and product-related studies (**4.3**).

In a first cluster of projects, we aimed to determine genomic characteristics of *S. aureus* from SFP outbreaks and from different sources (**section 4.1**), in order to identify the most common sources of staphylococcal food poisoning. To this end, *S. aureus* linked to outbreaks were compared to isolates from humans (cases of infections, nasal colonization), carcasses at slaughter (pigs, rabbits, poultry), milk (bovine, ovine, caprine), and ready-to-eat foods.

In a second cluster of projects, we aimed to determine the effect of different food-related stressors and regulatory mutations Δ`agr`, Δ`sarA`, Δ`sigB` on temporal enterotoxin D expression (**section 4.2**). To this end, we aimed to establish and use a quantitative Real-Time PCR system for quantification of gene expression under NaCl, nitrite, lactic acid, and glucose stress conditions. We created regulatory knockouts and assessed the effect of loss of these regulatory elements on enterotoxin expression during different growth phases in several enterotoxigenic strains.

In a third cluster of projects, we aimed to generate a deeper understanding for the factors leading to SFP outbreaks and the enterotoxins involved through outbreak investigations and product-related studies (**section 4.3**). To this end, we aimed to identify the causative agent of the respective outbreaks, to determine the original source and the causative enterotoxin, and to draw conclusions for risk mitigation. We also aimed to utilize this knowledge to adapt production processes with the goal of inhibiting *S. aureus* growth and enterotoxin formation.

4 S. aureus genomic characteristics

4.1 Publication 1

Noncontiguous finished genome sequence of *Staphylococcus aureus* KLT6, a staphylococcal enterotoxin B-positive strain involved in a food poisoning outbreak in Switzerland

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Noncontiguous Finished Genome Sequence of *Staphylococcus aureus* KLT6, a Staphylococcal Enterotoxin B-Positive Strain Involved in a Food Poisoning Outbreak in Switzerland

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We present the first complete genome sequence of a *Staphylococcus aureus* strain assigned to clonal complex 12. The strain was isolated in a food poisoning outbreak due to contaminated potato salad in Switzerland in 2009, and it produces staphylococcal enterotoxin B.

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Staphylococcus aureus not only represents a commensal that colonizes the nares of 20 to 30% of the global population (1), but it also causes severe infections, toxinoses, and life-threatening illnesses. Staphylococcal food poisoning is one of the most prevalent causes of food-borne intoxication worldwide. Shortly after intake of staphylococcal enterotoxins, patients exhibit violent emesis.

On 7 October 2009, ca. 400 people gathered for a yodeling festival in Münchwilen (Switzerland). Upon ingestion of contaminated potato salad, 30 participants suffered from acute vomiting and diarrhea. The outbreak was traced back to a staphylococcal enterotoxin B (SEB)-producing *S. aureus* strain that was designated strain KLT6 and was assigned to CC12 and *spa* type t160 (2).

We determined the genome sequence of KLT6 by combining optical mapping, long CCS (circular consensus sequencing) PacBio reads, and short Illumina reads.

A paired-end library of the KLT6 genome was created and sequenced using the Illumina HiSeq 2000 sequencer (GATC Biotech AG, Konstanz, Germany). Unspecified nucleotides (N) were removed and the 199,617,120 50-bp Illumina reads were *de novo* assembled using Velvet (3), resulting in 152 contigs. Optical mapping after digestion of KLT6 with NcoI and generation of PacBio reads using single-molecule real-time sequencing (SMRT) were outsourced to OpGen (Gaithersburg, MD) and Expression Analysis (Durham, NC). The assembly was refined to 31 contigs without any N, a maximum length contig of 1,275,455 bp and N_{60} of 313,040 bp, and one scaffold. The scaffolding was carried out using the optical map and the CCS PacBio reads (coverage, ~1×). Mummer and BLAST results of the alignment of the PacBio CCS and PacBio standard long reads to the contigs obtained with Velvet were used for scaffolding. A custom program based on graph databases was developed for connecting contigs, adding new sequence data from CCS at gaps when needed. Alignments to the regions of reference genomes (especially *S. aureus* NCTC 8325) exhibiting *in silico* restriction maps identical to those of KLT6 were also used in the final refinement of the assembly. RNA operons that appeared to be collapsed in the preliminary Velvet assembly were manually reconstructed by analyzing regions with significantly higher coverage than their neighboring sequences and by searching for compatible reads. The genome was annotated with BG7 (4) and contigs were connected when the joining of partial coding sequences located at the end of contigs could complete a specific protein.

One contiguous finished scaffold represents the complete circular KLT6 chromosome, consisting of 2,705,935 bp with a G+C content of 32.79%. The genome sequence of KLT6 contains 2,470 protein-coding genes, four complete 16S-5S-23S operons and 18 tRNA genes, ten noncoding RNAs, including an RNAIII regulatory transcript containing the delta hemolysin structural gene, eight riboswitches, a glucosamine-6-phosphate-activated ribozyme, and genes similar to bacteriophage phi-X174. Contigs KLT6000017 and KLT6000020 include 23 genes, many of them similar to the genes of plasmid pUSA300HOUMS in *S. aureus* USA300 TCH959.

Nucleotide sequence accession numbers. The sequence and annotation data of the *S. aureus* KLT6 genome were deposited in the GenBank database. This Whole-Genome Shotgun project was deposited at DDBJ/EMBL/GenBank under the accession no. APFH00000000. The version described in this paper is the first version, accession no. APFH01000000.

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4.2 Publication 2

Complete and assembled genome sequence of *Staphylococcus aureus* RKI4, a food-poisoning strain exhibiting a novel *S. aureus* pathogenicity island carrying *seb*

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Complete and Assembled Genome Sequence of *Staphylococcus aureus* RKI4, a Food-Poisoning Strain Exhibiting a Novel *S. aureus* Pathogenicity Island Carrying *seb*

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The genome of *Staphylococcus aureus* RKI4, a strain isolated from feces of a patient in a case of staphylococcal food poisoning, was sequenced using combined Illumina and single-molecule real-time sequencing. Hierarchical assembly of the genome resulted in a 2,725,654-bp chromosome and a 17,905-bp mobile genetic element.

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Staphylococcus aureus can cause staphylococcal food poisoning, with an estimated 240,000 cases occurring each year in the United States alone (1). Upon oral intake of staphylococcal enterotoxins, patients show signs of acute gastroenteritis, including violent vomiting and diarrhea. In this study, we present the complete genome sequence of *S. aureus* RKI4. The strain was isolated from feces of a 37-year-old patient suffering from staphylococcal food poisoning in Germany in 2008. The strain exhibits a novel *S. aureus* pathogenicity island (SaPI) carrying the *seb* gene encoding staphylococcal enterotoxin B. SaPIs are phage-related chromosomal islands and represent *S. aureus* mobile genetic elements. The novel SaPI described in this study exhibits the att site core 5′ ATT TTA CAT CAT TCC TGG CAT 3′. Production of enterotoxin B was confirmed using the SET-RPLA kit (Oxoid, Basel, Switzerland), and the strain was assigned to clonal complex 9 and *spa* type t733(2).

The genome of RKI4 was sequenced using a combination of Illumina HiSeq 2000 and PacBio single-molecule real-time sequencing (SMRT) technologies. The SMRT sequencing resulted in 104,146 reads with a mean length of 3,447 bp. The reads were assembled using the Hierarchical Genome Assembly Process (HGAP) (3). Duplicate sequences at the end of contigs were combined and the assembly resulted in two contigs of 2,725,653 and 17,905 bp, both with an average coverage of 102-fold. Illumina HiSeq 2000 sequencing resulted in 90,949,143 reads of 50-bp length. These reads were mapped to the 2 contigs obtained via HGAP using the CLC Genomics Workbench version 8.0 (CLC Bio, Aarhus, Denmark). A total of 88,702,834 reads could be mapped to contig 1, corresponding to a coverage of 1,627-fold. In addition, 2,390,850 reads were mapped to contig 2, resulting in a coverage of 6,627-fold. The Illumina reads were used to correct the SMRT reads, resulting in the complete assembled genome of *S. aureus* RKI4. The genome consists of a 2,725,654-bp chromosome and a 17,905-bp mobile genetic element with GC contents of 32.83 and 28.06%, respectively. Annotation was performed using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (4). RKI4 contains 2,631 predicted open reading frames, including 59 tRNA genes and 6 rRNA operons. The complete genome of RKI4 will contribute to further understanding of virulence and genome plasticity within *S. aureus*.

Nucleotide sequence accession numbers. Sequence and annotation data of the complete genome of *S. aureus* strain RKI4 were deposited in the GenBank database with the accession numbers CP011528 for the chromosome and CP011529 for the mobile genetic element.

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4.3 Publication 3

Comparison of virulence and antibiotic resistance genes of food poisoning outbreak isolates of *Staphylococcus aureus* with isolates obtained from bovine mastitis milk and pig carcasses

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Comparison of Virulence and Antibiotic Resistance Genes of Food Poisoning Outbreak Isolates of *Staphylococcus aureus* with Isolates Obtained from Bovine Mastitis Milk and Pig Carcasses

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ABSTRACT

Staphylococcus aureus is the etiological agent in a variety of infections in humans and livestock and produces enterotoxins leading to staphylococcal food poisoning (SFP), one of the most prevalent foodborne intoxication diseases worldwide. Pork and bovine milk are considered possible sources of SFP because pig skin is often colonized by *S. aureus* and bovine mastitis caused by *S. aureus* is common, but conclusive data are limited. The objective of the present study was to compare *S. aureus* isolates associated with cases of SFP with isolates obtained from bovine mastitis milk and pig carcasses. DNA microarray analysis and *spa* gene typing were performed with 100 *S. aureus* isolates: 20 isolates related to outbreaks of SFP in humans, 39 isolates obtained from pig carcasses, and 41 isolates collected from bovine mastitis milk. No overlap in *spa* types was observed for SFP isolates (t008, t015, t018, t024, t056, t084, t279, t377, t383, t648, t733, t912, t1239, t1270, t4802, and t6969) and isolates gathered from milk or pork. The porcine isolates were assigned to t034, t208, t337, t524, t899, t1939, t2922, t2971, t4475, and t7006, and the bovine isolates belonged to t267, t524, t529, t1403, t2953, t7007, t7008, and t7013. Comparison of microarray profiles revealed similar virulence gene patterns for isolates collected from the same host (pigs or cattle) but few similarities between SFP isolate profiles and the profiles of isolates obtained from bovine mastitis milk and pig carcasses. Although only some bovine and porcine isolates possessed the β-lactamase gene *blaZ* (milk, 24%; pork, 28%), significantly higher numbers of SFP isolates contained *blaZ* (90%). Investigations of these isolates provided no evidence that pork or bovine mastitis milk represent common sources of SFP.

Staphylococcus aureus is of major importance in human and veterinary medicine because it can cause a variety of symptoms and diseases, including toxic shock syndrome, septicemia, and skin, bone, and soft tissue infections. In the dairy industry, this pathogen is a common cause of bovine mastitis, leading to severe financial losses *(22)*. *S. aureus* is the etiological agent in a variety of infections in humans and livestock and produces enterotoxins leading to vomiting, diarrhea, and cramps upon ingestion. Staphylococcal food poisoning (SFP), one of the most prevalent foodborne intoxication diseases worldwide, results from consumption of enterotoxins that have formed in food *(16)*. Milk and pork are considered possible sources of SFP because colonization of pig skin and bovine mastitis caused by enterotoxigenic *S. aureus* are common, but conclusive data are limited *(4, 20, 28)*. Comparison of genetic polymorphisms in isolates gathered from various host species and isolates collected from individuals with SFP may allow evaluation of sources of strains associated with intoxication.

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The most widely used method for epidemiological investigation of *S. aureus* is *spa* typing. This typing method is based on sequence variations in the polymorphic X region of the *spa* gene, which encodes protein A. The X region consists of a variable number of 24-bp repeats flanked by well-conserved regions. *spa* typing allows highly discriminatory and rapid characterization and prediction of multilocus sequence types *(7, 14, 25)*. In a recent Swiss study, staphylococcal strains isolated from nasal passages of asymptomatic human volunteers were most frequently assigned to *spa* types t012 and t015, whereas t216 was common among *S. aureus* strains isolated from clinically infected human patients *(26)*. *spa* type t529 was reported as predominant among *S. aureus* isolates from bovine milk, and t034 was predominant among porcine *S. aureus* isolates *(9, 10, 18)*.

Recently, DNA microarrays have been used for genotyping of *S. aureus*; this technique permits rapid detection of many virulence genes and resistance determinants *(17, 18)*. The ArrayTube AT platform utilizes multiplex linear DNA amplification and microarray hybridization. The respective probes, which are present in duplicate on the microarray, have been described *(17, 18)*.

TABLE 1. *SFP isolates evaluated in this study*

Isolate	Sample	Source; no. of patients	Origin[a]
SFP1	Feces, food	Unskimmed milk; 2 children (ages 3 and 6 yr)	RKI
SFP2	Food	Milk, rice; group of children	RKI
SFP3	Feces, food	Meat, pancakes, etc.; group of adults	RKI
SFP4	Feces	ND[b]; 1 adult	RKI
SFP5	Feces	ND; 1 senior citizen (age 71 yr)	RKI
SFP6	Feces	ND; 1 child (age 4 yr)	RKI
SFP7	Feces	ND; ND	BAHFS
SFP8	Feces	ND; ND	BAHFS
SFP9	Feces	ND; ND	BAHFS
SFP10	Feces	ND; ND	BAHFS
SFP11	Feces	ND; ND	BAHFS
SFP12	Feces	ND; ND	BAHFS
SFP13	Feces	ND; ND	BAHFS
SFP14	Feces	ND; ND	BAHFS
SFP15	Feces	ND; ND	BAHFS
SFP16	Feces	ND; ND	BAHFS
SFP17	Feces	ND; ND	BAHFS
SFP18	Food	ND; ND	GFAF
SFP19	Food	ND; ND	GFAF
SFP20	Food	Potato salad; 18 children and 9 adults	CLF

[a] RKI, Robert Koch Institute; BAHFS, Bavarian Authorities for Health and Food Safety; GFAF, Medical Department of the German Federal Armed Forces; CLF, Cantonal Laboratory of Fribourg.
[b] ND, no data available.

This method allows detection of 170 genes, including species markers and controls and 144 target genes *(18)*.

In the present study, *spa* typing and DNA microarray analysis were performed with 100 *S. aureus* isolates to compare isolates associated with cases of SFP with isolates obtained from bovine mastitis milk and pig carcasses.

MATERIALS AND METHODS

Bacterial isolates. The 100 *S. aureus* isolates investigated included 20 isolates related to outbreaks of SFP in humans, 39 porcine isolates, and 41 bovine mastitis isolates. The 20 *S. aureus* isolates used in this study that were associated with outbreaks of SFP were collected by the German National Reference Center for Staphylococci (Robert Koch Institute, Wernigerode, Germany), the Bavarian Authorities for Health and Food Safety (Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Munich, Germany), the Medical Department of the German Federal Armed Forces (Zentrales Institut des Sanitätsdienstes der Bundeswehr Kiel, Kronshagen, Germany), and the Cantonal Laboratory of Fribourg (Fribourg, Switzerland) (Table 1). The porcine isolates were obtained from animals that had originated from farms throughout Switzerland and had been slaughtered in two Swiss abattoirs during a period of 10 months. Samples were collected using the wet-dry double swab technique from neck, belly, back, and ham of pig carcasses before scalding. The bovine mastitis isolates were obtained from the milk of 41 cows. The animals belonged to 38 different herds located throughout the German-speaking part of Switzerland.

DNA extraction, species identification, and DNA purification. Kits for DNA isolation and purification were obtained from QIAGEN (Hilden, Germany) and handled following the manufacturer's instructions. The DNeasy Blood and Tissue Kit was used to isolate chromosomal DNA from stationary phase cultures that had been grown for 16 to 18 h in brain heart infusion (Oxoid, Basingstoke, UK) at 37°C under agitation (220 rpm). Identification of presumptive *S. aureus* isolates was confirmed using a species-specific PCR assay targeting the 16S–23S rRNA intergenic spacer region with the Staur 4 and 6 primers as described previously *(24)*. All primers were synthesized by Microsynth (Balgach, Switzerland), and PCR ingredients were supplied by Promega (Madison, WI) unless otherwise indicated. DNA fragments from PCRs were purified using the MinElute PCR Purification Kit. The concentration of nucleic acids was determined using an ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

***spa* typing.** The sequence of the polymorphic X region of the *spa* gene of each *S. aureus* isolate was determined as described by Aires-de-Sousa et al. *(1)* with minor modifications. The *spa* sequence was amplified with spa-1113f and spa-1514r primers using the GoTaq PCR system (Promega AG, Dübendorf, Switzerland) under the following reaction conditions: (i) 5 min at 94°C; (ii) 35 cycles of 45 s at 94°C, 45 s at 60°C, and 90 s at 72°C; and (iii) 10 min at 72°C. PCR amplicons were purified, and sequencing was outsourced (Microsynth). The sequences were assigned to *spa* types using the *spa*-server (http://www.spaserver.ridom.de/) *(8)*. Clonal complexes were determined using Ridom StaphType 2.0.3 software and the based-upon-repeat-pattern algorithm. The sequences of the polymorphic X region of *spa* were compared, and a minimum spanning tree was created using BioNumerics 6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Microarray-based genotyping. Staphylococcal DNA was genotyped using an ArrayTube-based DNA microarray approach (Clondiag Chip Technologies, Jena, Germany) according to the manufacturer's instructions. Average signal intensities of control spots and species markers were calculated, and only runs with values greater than 0.4 were considered valid. Single spots with a

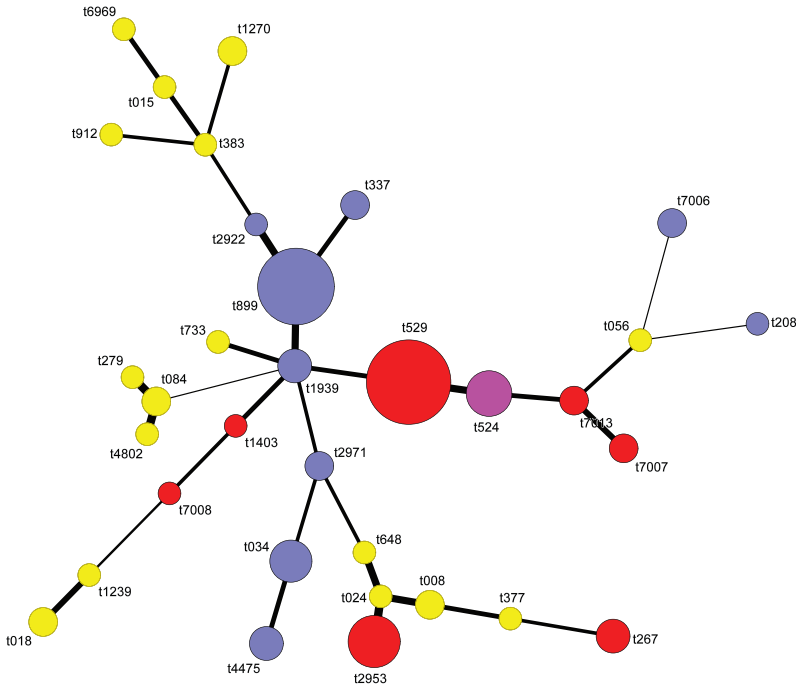

FIGURE 1. *Minimum spanning tree visualizing the* spa *typing results. Each node represents a* spa *type; the size of the circle represents the number of isolates assigned to each* spa *type, and branch thickness indicates distance. Node colors refer to the source of the isolates allocated to the* spa *type (red, bovine mastitis milk; purple, pork; yellow, SFP; pink,* spa *types with both porcine and bovine isolates).*

normalized signal intensity greater than 50% of the average signal intensity of control spots and species markers of the same run were defined as positive or present, spots with signal intensities between 33 and 50% of this value were defined as ambiguous, and spots with signal intensities below 33% were considered negative or absent. Similar to Coombs et al. *(6),* profiles were compared using SplitsTree4, a software package designed to compute unrooted phylogenetic networks from molecular sequence data *(11).* DNA microarray gene profiles were converted to sequence-like strings of information, defining present genes as A (positive), absent genes as T (negative), and spots with ambiguous signal intensities as missing.

Statistical analysis. The distribution of genes among bovine, porcine, and SFP isolates was compared based on the hybridization results of the DNA microarray. PASW 18 (SPSS Inc., Chicago, IL) was used to perform Pearson's chi-square test, determining significant associations between the source of the isolates (SFP, milk, or pork) and the presence of the examined genes. Results were considered significant at $P < 0.050$.

TABLE 2. spa *types and predicted clonal complexes of bovine, porcine, and SFP isolates investigated in this study*

Isolate origin	Clonal complex (% or no. of isolates assigned to that type)	*spa* type(s) (no. of isolates assigned to that type)
Milk	CC8 (17%)	t2953 (7)
	CC25 (5%)	t7007 (2)
	CC97 (15%)	t267 (3), t524 (3)
	CC133 (2%)	t1403 (1)
	CC151 (54%)	t529 (22)
	CC[a] (7%)	t7008 (1), t7013 (2)
Pork	CC5 (1)	t7006 (1)
	CC9 (23)	t337 (2), t899 (18), t1939 (3)
	CC49 (1)	t208 (1)
	CC97 (3)	t524 (3)
	CC398 (11)	t034 (5), t2922 (1), t2971 (2), t4475 (3)
SFP	CC8 (5)	t008 (2), t024 (1), t337 (1), t648 (1)
	CC9 (1)	t733 (1)
	CC15 (4)	t084 (2), t279 (1), t4802 (1)
	CC22 (1)	t1239 (1)
	CC30 (2)	t018 (2)
	CC45 (4)	t383 (1), t1270 (2), t6969 (1)
	CC73 (1)	t015 (1)
	CC101 (1)	t056 (1)
	CC[a]	t912 (1)

[a] No clonal complex could be assigned.

TABLE 3. *Detection of antibiotic resistance genes in* S. aureus *isolates collected from human cases of SFP, bovine mastitis milk, and pig carcasses*[a]

Gene[b]	Affected antibiotic(s)	% isolates positive for each gene			*P* value (Pearson's chi-square test)
		SFP ($n = 20$)	Milk ($n = 41$)	Pork ($n = 39$)	
mecA	Methicillin	0	0	0	≥0.050
blaZ	Beta-lactams	90[c]	24	28	0.000 (SFP, milk), 0.000 (SFP, pork)
ermC	Macrolides, lincosamides, streptogramin	5	2	0	≥0.050
aadD	Kanamycin, neomycin, tobramycin	0	2	3	≥0.050
far1	Fusidic acid	5	0	0	≥0.050
tetK	Tetracycline	10	0	5	≥0.050
tetM	Tetracycline	0	5	21[c]	0.027 (SFP, pork), 0.031 (SFP, milk)
vanB	Vancomycin	0	2	0	≥0.050

[a] Genes were determined to be present based on DNA microarray analysis. Calculations include positive signals only; ambiguous signals were omitted.
[b] Resistance genes not detected among isolates from the three sources: *vanA/Z, ermA/C, linA, msrA, vatA/B, vga/b, aacA-aphD, sat, dfrA, aphA3,* and *mupR*.
[c] Result for this source differs significantly from the result calculated for both other sources of isolates investigated in this study ($P < 0.050$).

RESULTS

***spa* typing.** The 100 *S. aureus* isolates investigated in this study were categorized into 33 different *spa* types and assigned to 15 clonal complexes (Fig. 1). Although 29 *spa* types were assigned to clonal complexes, three *spa* types could not be assigned (Table 2). The 41 isolates obtained from bovine mastitis milk were grouped into clonal complexes CC8 (t2953), CC25 (t7007), CC97 (t267 and t524), CC133 (t1403), and CC151 (t529); types t7008 and t7013 in this group could not be assigned. The 39 isolates obtained from pig carcasses were assigned to CC5 (t7006), CC9 (t337, t899, and t1939), CC49 (t208), CC97 (t524), and C398 (t034, t2922, t2971, and t4475); a minor overlap

TABLE 4. *Detection of genes encoding superantigenic toxins in* S. aureus *isolates collected from human cases of SFP, bovine mastitis milk, and pig carcasses*[a]

Gene[b]	% isolates positive for each gene			*P* value (Pearson's chi-square test)
	SFP ($n = 20$)	Milk ($n = 41$)	Pork ($n = 39$)	
entA	35[c]	12[c]	0[c]	0.040 (SFP, milk), 0.000 (SFP, pork), 0.023 (milk, pork)
entB	0	17[c] (pork)	0[c] (milk)	0.007 (milk, pork)
entC	15	22	0[c]	0.013 (SFP, pork), 0.002 (milk, pork)
entD	15	27	0[c]	0.013 (SFP, pork), 0.000 (milk, pork)
entE	0	0	0	≥0.050
entG	45	63	67	≥0.050
entH	0	0	0	≥0.050
entI	40	46	67	≥0.050
entJ	15	17	0[c]	0.013 (SFP, pork), 0.007 (milk, pork)
entL	15	22	0[c]	0.013 (SFP, pork), 0.002 (milk, pork)
entM	45	17[c] (pork)	67[c] (milk)	0.000 (milk, pork)
entN	45	10[c]	62	0.000 (milk, pork), 0.002 (SFP, milk)
entO	35	51	64	≥0.050
entR	15	15	0[c]	0.013 (SFP, pork), 0.012 (milk, pork)
entX	100	100	100	≥0.050
entY	55	66	85[c]	0.000 (SFP, pork), 0.010 (milk, pork)
tst	20[c] (pork)	5	0[c] (SFP)	0.004 (SFP, pork)

[a] Genes were determined to be present based on DNA microarray analysis. Calculations include positive signals only; ambiguous signals were omitted.
[b] Genes *entA* through *entY* encode staphylococcal enterotoxins; gene *tst* encodes toxic shock syndrome toxin 1.
[c] Result for this source differs significantly from the result calculated for one (source indicated) or both other sources of isolates ($P < 0.050$).

TABLE 5. *Detection of other virulence determinants, including genes encoding leukocidins, hemolysins, and staphylokinase, in* S. aureus *isolates collected from human cases of SFP, bovine mastitis milk, and pig carcasses*[a]

Gene	Protein	% isolates positive for each gene			*P* value (Pearson's chi-square test)
		SFP ($n = 20$)	Milk ($n = 41$)	Pork ($n = 39$)	
lukD	Leukocidin D	55[b]	98[b]	10[b]	0.000 (SFP, milk), 0.000 (SFP, pork), 0.000 (milk, pork)
lukE	Leukocidin E	30[b]	71[b]	5[b]	0.002 (SFP, milk), 0.007 (SFP, pork), 0.000 (milk, pork)
lukF	Leukocidin subunit F (B)[c]	100	100	100	≥0.050
lukM	Hypothetical protein (lukS-PV-P83)	0	73[b]	3	0.000 (SFP, milk), 0.000 (milk, pork)
lukS	Leukocidin subunit S (C)[c]	80[b]	100	100	0.003 (SFP, milk), 0.004 (SFP, pork)
lukX	Leukocidin X	40	12[b]	46	0.013 (SFP, milk), 0.000 (milk, pork)
hl	Hypothetical protein similar to hemolysin	100	100	100	≥0.050
hla	Alpha-hemolysin toxin	80[b]	100	100	0.003 (SFP, milk), 0.004 (SFP, pork)
hlb	Beta-hemolysin toxin	30[b]	80	82	0.000 (SFP, milk), 0.000 (SFP, pork)
hld	Delta-hemolysin toxin	90[b]	100	100	0.040 (SFP, milk), 0.045 (SFP, pork)
hlgA	Gamma-hemolysin toxin (A)[c]	100	98	100	≥0.050
hl-III	Putative hemolysin III	75[b]	32[b]	97[b]	0.001 (SFP, milk), 0.007 (SFP, pork), 0.000 (milk, pork)
sak	Staphylokinase	60[b]	12	3	0.000 (SFP, milk), 0.000 (SFP, pork)

[a] Genes were determined to be present based on DNA microarray analysis. Calculations include positive signals only; ambiguous signals were omitted.

[b] Result for this source differs significantly from the result calculated for both other sources of isolates investigated in this study ($P < 0.050$).

[c] Component encoded by respective gene is indicated in parentheses.

with the bovine mastitis milk isolates occurred with three isolates each in CC97 (t524). The 20 isolates associated with human cases of SFP were assigned to CC8 (t008, t024, t337, and t648), CC9 (t733), CC15 (t084, t279, and t4802), CC22 (t1239), CC30 (t018), CC45 (t383, t1270, and t6969), CC73 (t015), and CC101 (t056); type t912 in this group could not be assigned. Although we observed overlap in clonal complexes between SFP isolates and those from milk (CC8) and pork (CC9), the *spa* types assigned to SFP isolates were not found in either animal source.

DNA microarray. Distribution of virulence genes among isolates collected from bovine mastitis milk, pig carcasses, and human cases of SFP was investigated by DNA microarray analysis. Few isolates possessed genes involved in antibiotic resistance; the resistance genes *blaZ, ermC, aadD, tetK*/*M,* and *vanB* were detected (Table 3). The genes *mecA, ermA, linA, msrA, vatA*/*B, vga*/*b, aacA-aphD, aphA3, sat, dfrA, mupR,* and *vanA*/*Z* were not found. When *S. aureus* isolates collected from different sources were compared, a significantly higher number of SFP than bovine and porcine isolates possessed *blaZ* ($P = 0.000$) and significantly more porcine isolates than both SFP and bovine mastitis milk isolates possessed *tetM,* which is involved in resistance to tetracyclines (SFP, $P = 0.027$; milk, $P = 0.031$).

Findings regarding superantigenic toxins are displayed in Table 4. Although *entX* was present in all investigated *S. aureus* isolates, *entE* and *entH* were not detected in any isolate. The porcine strains also lacked other classical enterotoxin genes and the genes *entJ, entL, entR,* and *tst* (encoding toxic shock syndrome toxin 1), which were found in bovine milk and SFP strains.

The distribution of other virulence determinants, including genes encoding leukocidins, hemolysins, and

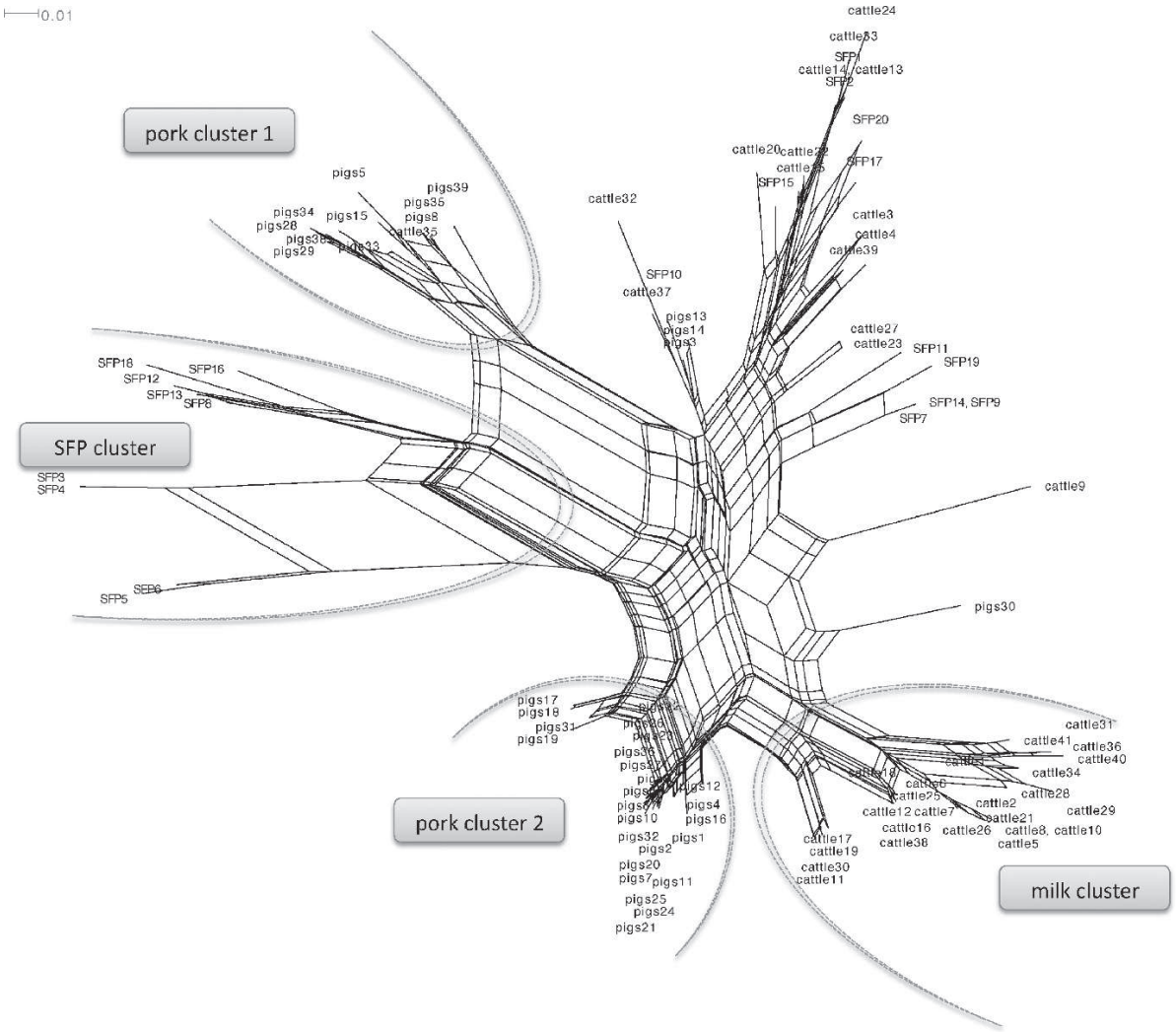

FIGURE 2. *SplitsTree visualizing the similarity among gene profiles obtained by DNA microarray analysis for the 100* S. aureus *isolates investigated. Four prominent clusters were observed that almost exclusively contained isolates from the same source.*

staphylokinase, is given in Table 5. The *lukM* gene was present in a significantly higher number of bovine mastitis milk isolates (73%) than in both porcine (3%) and SFP (0%) isolates ($P = 0.000$). The *sak* gene, which codes for staphylokinase (a bacteriophage-encoded protein that is expressed by lysogenic *S. aureus* strains), exhibited significantly higher prevalence ($P = 0.000$) in isolates associated with SFP (60%) than in isolates obtained from milk (12%) or pork (3%) *(3)*.

All microarray profiles were analyzed for similarities by construction of the SplitsTree shown in Figure 2. Four prominent clusters almost exclusively contained isolates of the same source. The SFP cluster consisted of nine SFP isolates, and the milk cluster consisted of 25 bovine mastitis milk isolates. Pork clusters 1 and 2 consisted of 10 and 25 porcine isolates, respectively; pork cluster 1 also containing one bovine isolate. Several smaller source-specific and a few mixed clusters also were identified.

DISCUSSION

Comparison of clonal complexes of SFP isolates with those of milk and pork isolates revealed that isolates tended to cluster depending on their source into identical clonal complexes and *spa* types, with little overlap. In recent studies conducted in Switzerland and Germany, CC8, CC15, CC30, and CC45 were the most common clonal complexes among staphylococcal isolates obtained from asymptomatic human carriers and individuals with clinical staphylococcal infections *(19, 26)*. Clonal complexes CC8 (t008, t024, t337, and t648), CC15 (t084, t279, and t4802), CC30 (t018), and CC45 (t383, t1270, and t6969) were also predicted for SFP isolates in our study, including *spa* types t008 and t377, which were rarely present among bovine milk isolates in a comprehensive Japanese study *(10)*. A few SFP isolates also were assigned to t084 and t015, *spa* types reported in methicillin-sensitive *S. aureus* strains causing infections in humans *(15, 27)*.

Isolates obtained from bovine mastitis milk clustered into CC151 (22 isolates of type t529), CC8 (7 isolates of t2953), and CC97 (6 isolates of t267 and t524), and most porcine isolates were grouped into CC9 (23 isolates of t899, t337, and t1939). In recent studies on isolates collected in Switzerland, Germany, and Japan, t529 was the predominant type among *S. aureus* isolates obtained from bovine milk *(10, 18)*. In Denmark, clonal complexes CC97 (t524), CC50 (t518), and CC151 (t529) were predominant among bovine isolates, and CC398 (t034), CC30 (t1333), and CC9 (t337) were predominant among porcine isolates *(9)*.

Consistent with the findings in our study, Monecke et al. *(18)* reported a high prevalence of *lukF-P83*/*lukM* (a bicomponent leukocidin that was suggested to contribute to mastitis in cows) in bovine *S. aureus* isolates *(21)*. Also in accord with our findings, a high prevalence of *sak* in human *S. aureus* strains and a low prevalence of *sak* among cattle isolates was described *(18)*.

In bovine and SFP isolates, genes encoding superantigenic toxins were mostly present in similar numbers. The porcine isolates did not possess any classical enterotoxin genes or *tst*. Genes encoding staphylococcal enterotoxins A and C were found in a study of raw pork and uncooked smoked ham *(2)*. However, contamination may occur during meat handling and processing, and currently no comparable data are available on the prevalence of enterotoxin genes in *S. aureus* isolates obtained from pig carcasses. Regarding the bovine mastitis isolates, our results resemble those of other researchers who have investigated the presence of enterotoxin genes in bovine *S. aureus*. Compared with Monecke et al. *(18),* we found lower numbers of *entM*-producing isolates. In other studies conducted in Germany, Japan, and the United States, lower prevalences of classical enterotoxin genes have been reported *(12, 23, 28)*. In comparison with our results regarding prevalence rates of enterotoxin genes in SFP isolates, in a Taiwanese study higher numbers of *entB*-producing *S. aureus* isolates were found, but the prevalence of *entC, entD,* and several newly described enterotoxin genes was low *(5)*. In a French study, *entA* was the most prevalent enterotoxin gene, followed by *entD, entG, entI,* and *entH (13)*.

Comparison of microarray profiles revealed a high degree of similarity between virulence gene patterns for isolates collected from the same source but few similarities between SFP profiles and profiles obtained from bovine milk and pork isolates. We also found no overlap in *spa* types for SFP isolates and isolates collected from bovine mastitis milk and pig carcasses. On the contrary, isolates tended to group into predominantly source-specific *spa* types and microarray profile clusters, thus indicating relative host specificity. Based on the isolates investigated in this study, we hypothesize that neither bovine mastitis milk nor pork represent common sources of SFP.

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4.4 Publication 4

Comparison of *Staphylococcus aureus* isolates associated with food intoxication with isolates from human nasal carriers and human infections

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ARTICLE

Comparison of *Staphylococcus aureus* isolates associated with food intoxication with isolates from human nasal carriers and human infections

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Abstract *Staphylococcus aureus* represents an organism of striking versatility. While asymptomatic nasal colonization is widespread, it can also cause serious infections, toxinoses and life-threatening illnesses in humans and animals. Staphylococcal food poisoning (SFP), one of the most prevalent causes of foodborne intoxication worldwide, results from oral intake of staphylococcal enterotoxins leading to violent vomiting, diarrhea and cramps shortly upon ingestion. The aim of the present study was to compare isolates associated with SFP to isolates collected from cases of human nasal colonization and clinical infections in order to investigate the role of *S. aureus* colonizing and infecting humans as a possible source of SFP. *Spa* typing and DNA microarray profiling were used to characterize a total of 120 isolates, comprising 50 isolates collected from the anterior nares of healthy donors, 50 isolates obtained from cases of clinical infections in humans and 20 isolates related to outbreaks of staphylococcal food poisoning. Several common *spa* types were found among isolates of all three sources (t015, t018, t056, t084). DNA microarray results showed highly similar virulence gene profiles for isolates from all tested sources. These results suggest contamination of foodstuff with *S. aureus* colonizing and infecting food handlers to represent a source of SFP.

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Introduction

Staphylococcus aureus is not only a commensal colonizer, but can also cause serious infections, toxinoses and life-threatening diseases, such as skin and soft tissue infections, toxic shock syndrome and septicemia. *S. aureus* colonizes skin and mucosa of humans and animals, with nasal carriage rates between 30% and 50% among the adult human population [1–4]. While colonization of the anterior nares is usually asymptomatic, it serves as a reservoir for the spread of the organism [1, 5]. Carriers are at increased risk to develop nosocomial bacteremia which in 80% of cases is caused by the strain colonizing their nares [6, 7]. The rapid emergence of antibiotic resistance among *S. aureus* is also known to play a crucial role in the epidemiology of staphylococcal infections. Recently, infections with methicillin resistant *S. aureus* (MRSA) have been estimated to constitute the leading cause of death due to one single infectious agent in the United States [8].

S. aureus also represents the cause of staphylococcal food poisoning (SFP), one of the most prevalent foodborne intoxications worldwide. SFP results from ingestion of staphylococcal enterotoxins preformed in food, typically presenting with violent emesis, nausea, diarrhea and prostration. While in most cases symptoms subside spontaneously after 24 h, fatality rates range from 0.03% in the general population to 4.4% in children and the elderly [9]. As staphylococcal colonization and infection is widely spread, contamination of foodstuff by food handlers may represent a major source of SFP. As SFP isolates are difficult to obtain, to date, there is only very limited information on the original source of enterotoxigenic *S. aureus* strains that lead to cases of food poisoning.

Different techniques are established for typing *S. aureus*. The most widely used method for epidemiological inves-

tigations is *spa* typing, based on the determination of the polymorphic X region of the gene encoding staphylococcal protein A (*spa*). DNA microarray is used for rapid detection of a multitude of virulence genes (genes encoding enterotoxins, hemolysins, leukocidins, etc.), resistance determinants, and typing markers. The resulting hybridization pattern can be used to assign isolates to clonal complexes [10].

In this study, *spa* typing and DNA microarray analysis were performed with a total of 120 *S. aureus* isolates, comprising *S. aureus* isolates obtained from nasal colonization in healthy donors, isolates gained from clinical cases of infection and isolates associated with outbreaks of staphylococcal food poisoning. The objective was to compare SFP isolates to isolates obtained from *S. aureus* nasal colonization (SANC) and clinical cases of infection (SAI) in order to determine the role of *S. aureus* colonizing and infecting humans as a possible source of SFP.

Materials and methods

Bacterial isolates

A total of 120 *S. aureus* isolates were examined, constituting 50 SANC isolates, 50 SAI isolates and 20 isolates associated to outbreaks of SFP in humans. Nasal swabs of the anterior nares were collected from randomly chosen volunteers in Switzerland between November and December 2010. Samples from both nostrils were taken using sterile cotton swabs moistened with saline. Fifty SAI isolates were obtained from the Institute of Medical Microbiology of the University of Zurich, Switzerland, between November and December 2010. The 20 SFP isolates were provided by the Bavarian Authorities for Health and Food Safety (LGL, Munich, Germany), the German National Reference Center for Staphylococci (Robert Koch Institute, Wernigerode, Germany), the Cantonal Laboratory Fribourg (Fribourg, Switzerland) and the Medical Department of the German Federal Armed Forces (Kronshagen, Germany) (Table 1). Ethical clearance was granted by the locally cognizant ethics commission (cantonal ethics commission, Zurich).

DNA extraction and species identification

Swabs were streaked directly onto rabbit plasma fibrinogen (RPF) plates (Oxoid, Basel, Switzerland), incubated at 37°C and examined for coagulase activity after 48 h. Two *S. aureus* typical colonies (colonies surrounded by an opaque halo) each were subcultured on RPF plates (48 h at 37°C). One colony of each plate was transferred to blood agar and incubated overnight at 37°C. DNA isolation kits were obtained from QIAGEN (Hilden, Germany) and handled according to the manufacturer's instructions. The PCR consumables were supplied by Promega (Madison, Wisconsin,

Table 1 Staphylococcal food poisoning (SFP) isolates included in this study

ID	Sample	Institution providing the isolate
SFP1	Food	Medical Department of the German Federal Armed Forces
SFP2	Food	Medical Department of the German Federal Armed Forces
SFP3	Food	Cantonal Laboratory of Fribourg
SFP4	Feces	Bavarian Authorities for Health and Food Safety
SFP5	Feces	Bavarian Authorities for Health and Food Safety
SFP6	Feces	Bavarian Authorities for Health and Food Safety
SFP7	Feces	Bavarian Authorities for Health and Food Safety
SFP8	Feces	Bavarian Authorities for Health and Food Safety
SFP9	Feces	Bavarian Authorities for Health and Food Safety
SFP10	Feces	Bavarian Authorities for Health and Food Safety
SFP11	Feces	Bavarian Authorities for Health and Food Safety
SFP12	Feces	Bavarian Authorities for Health and Food Safety
SFP13	Feces	Bavarian Authorities for Health and Food Safety
SFP14	Feces	Bavarian Authorities for Health and Food Safety
SFP15	Feces+food	Robert Koch Institute
SFP16	Food	Robert Koch Institute
SFP17	Feces+food	Robert Koch Institute
SFP18	Feces	Robert Koch Institute
SFP19	Feces	Robert Koch Institute
SFP20	Feces	Robert Koch Institute

USA). The DNA concentration was measured by using a Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Spa typing

The sequence of the polymorphic X region of the *spa* gene of each *S. aureus* isolate was determined as described by Aires-de-Sousa et al. [11], with minor modifications. Briefly, the *spa* gene was amplified with spa-1113f and spa-1514r primers (Table 2) using the GoTaq PCR system (Promega AG, Dübendorf, Switzerland) at the following reaction conditions: (i) 5 min at 94°C; (ii) 35x [45 s at 94°C; 45 s at 60°C; 90 s at 72°C]; (iii) 10 min at 72°C. PCR purification and sequencing was outsourced (GATC Biotech, Constance, Germany and Microsynth, Balgach, Switzerland). The sequences were assigned to *spa* types using the *spa*-server (http://www.spaserver.ridom.de/) [12]. Clonal complexes were determined using Ridom StaphType 2.0.3 software and the Based Upon Repeat Pattern (BURP) algorithm.

Microarray based genotyping

For DNA microarray profiling the StaphyType ArrayStrip platform was used according to the manufacturer's instructions (Clondiag chip technologies, Jena, Germany). Similar to Coombs et al., microarray profiles were compared using SplitsTree4, a software designed to compute unrooted phylogenetic networks from molecular sequence data [13, 14]. DNA microarray gene profiles were converted to "sequence-like" strings of information, defining present genes as "A" (positive), absent genes as "T" (negative) and spots with ambiguous signal intensities as missing.

Statistical analysis

The distribution of genes among SANC, SAI, and SFP isolates was compared based on the hybridization results of the DNA microarray. SPSS Statistics 19 was used to run Pearson's chi-squared test, identifying significant associations between the source the isolates were collected from and the presence of the examined genes. *P*-values<0.05 were considered statistically significant.

Results

Screening of nasal swabs for the presence of *S. aureus* showed a nasal carriage rate of 37.6% among the 133 healthy test persons.

The 120 staphylococcal isolates tested, including 50 SANC, 50 SAI, as well as 20 SFP isolates, could be assigned to 20 clonal complexes comprising a total of 79 different *spa* types (see Table 3). Among SANC, SAI, and SFP isolates, clonal complexes CC8, CC15, CC30, CC45, CC78, and CC101 could be found. Isolates from all three sources were frequently assigned to CC45 (SANC: 16%, SAI: 20%, SFP: 30%). While high prevalence of CC30 was found among SANC (24%) and SFP isolates (15%), SAI isolates were more often assigned to CC59 (14%). The 50 isolates from nasal swabs were grouped into 39 *spa* types with *spa* type t015 and t012 being found most frequently (8% each). The 50 SAI isolates grouped into 38 different *spa* types, with t216 representing the most common *spa* type (12%). The 20 isolates associated with SFP were grouped into 15 *spa* types. Some common *spa* types were found among isolates of all three sources (t015, t018, t056, and t084). Isolates obtained from nasal colonization and cases of clinical infections were overlapping in *spa* types t002, t127, t148, and t216. Spa type t008 was found in both clinical and food poisoning isolates of *S. aureus*.

DNA microarray was used to determine gene profiles of all 120 strains. Hybridization results for *agr* and capsule types are depicted in Table 4. While *agrI* was found to represent the most frequent *agr* type among all three sources, SANC and SAI isolates differed significantly in the number of isolates assigned to *agrI* (36% SANC, 70% SAI; $p=0.001$) and *agrIII* (28% SANC, 8% SAI, $p=0.009$).

Most isolates possessed one or several genes involved in resistance to antimicrobial agents (see Table 5). The *blaZ* gene conferring resistance to beta lactams was found most frequently among isolates from all three sources (SANC: 74%, SAI: 76%, SFP: 85%). Antibiotic resistance profiles were highly similar for SANC, SAI, and SFP isolates. Only *fosB*, which is involved in resistance to fosfomycin and bleomycin, was present in significantly higher numbers in SANC than in SAI isolates (SANC: 68%, SAI: 38%; $p=0.002$). While genes involved in vancomycin resistance (*vanA/B/Z*) were not found, few isolates exhibited genes

Table 2 Primers used in this study

Name	Nucleotide sequence (5′ → 3′)	Product size	Reference
spa-1113f	5′ TAA AGA CGA TCC TTC GGT GAG C 3′	Variable	[11]
spa-1514r	5′ CAG CAG TAG TGC CGT TTG CTT 3′		
Staur 4	5′ ACG GAG TTA CAA AGG ACG AC 3′	1250 bp	[31]
Staur 6	AGC TCA GCC TTA ACT AGC AG 3′		

Table 3 *Spa* types and predicted clonal complexes of *S. aureus* nasal colonization (SANC), clinical cases of infection (SAI) and staphylococcal food poisoning (SFP) isolates investigated in this study

Clonal complex	*Spa* type	Numerical code assigned to repeats	SANC(n=50)	SAI(n=50)	SFP(n=20)
CC1	t127	07-23-21-16-34-33-13	1	1	0
	t189	07-23-12-21-17-34	0	1	0
	t8021	07-23-12-21-23-12-21-17-34	0	1	0
CC5	t002	26-23-17-34-17-20-17-12-17-16	2	3	0
	t010	26-17-34-17-20-17-12-17-16	1	0	0
	t105	26-23-17-34-17-20-17-17-16	1	0	0
	t857	26-23-17-34-17-13-17-16	1	0	0
	t954[a]	26-23-17-34-17-17-16	0	1	0
	t1062	26-23-17-34-17-02-17-12-17-16	1	0	0
	t8017	35-17-34-17-20-17-17-12-17-16	0	1	0
	t8020	07-22-17-20-17-12-17-17-16-16	1	0	0
CC6	t701	11-10-21-17-34-24-34-22-25-25	0	1	0
CC7	t091	07-23-21-17-34-12-23-02-12-23	0	2	0
CC8	t008	11-19-12-21-17-34-24-34-22-25	0	1	2
	t024	11-12-21-17-34-24-34-22-25	0	0	1
	t148	07-23-12-21-12-17-20-17-12-12-17	1	1	0
	t334	11-12-21-17-34-22-25	1	0	0
	t648	11-21-17-34-24-34-22-25	0	0	1
	t8016	07-23-13-21-22-34-34-34-34-33-34	1	0	0
CC9	t209	07-16-12-23-34	1	0	0
	t733	26-23-02-12-23-02-34-34-34	0	0	1
CC12	t156	07-06-17-21-34-34-22-34	0	1	0
	t5444	14-12-33-22-17	1	0	0
CC15	t084	07-23-12-34-34-12-12-23-02-12-23	3	3	2
	t085	07-23-12-34-34-12-23-02-12-23	0	1	0
	t279	07-23-12-34-34-34-12-12-23-02-12-23	0	0	1
	t328	07-23-12-34-34-12-12-23-02-12-23-02-12-23	1	0	0
	t529	04-34	1	0	0
	t774	07-23-12-34-34-12-12-12-23-02-12-23	0	1	0
	t1038	07-23-12-34-13-12-12-23-02-12-23	1	0	0
	t4802	07-23-12-34-34-12-12-23-23-02-12-23	0	0	1
CC20	t164	07-06-17-21-34-34-22-34	0	1	0
CC22	t005	26-23-13-23-31-05-17-25-17-25-16-28	0	1	0
	t310	26-23-31-05-17-25-17-25-16-28	0	1	0
	t852	07-23-13-23-31-05-17-25-17-25-16-28	0	1	0
	t8019	26-23-13-23-31-05-17-25-17-24-16-28	0	1	0
CC25	t349	04-21-12-17-20-17-12-12-17	1	0	0
CC30	t012	15-12-16-02-16-02-25-17-24-24	4	0	0
	t017	15-12-16-16-02-16-02-25-17-24-24	1	0	0
	t018[a]	15-12-16-02-16-02-25-17-24-24-24	1	1	2
	t021	15-12-16-02-16-02-25-17-24	2	0	0
	t338	15-21-16-02-25-17-24	1	0	0
	t8455	15-12-16-16-34-02-16-02-25-17-24-24	1	0	0
	t1076	04-12-25-22-34	1	0	0
	t1239	15-12-16-02-16-02-24-24-24	0	0	1
	t4516	15-12-16-24-24	1	0	0
CC45	t015[a]	08-16-02-16-34-13-17-34-16-34	4	4	1
	t050	08-16-02-16-34-34-17-34-16-34	1	0	0

Table 3 (continued)

Clonal complex	*Spa* type	Numerical code assigned to repeats	SANC(n=50)	SAI(n=50)	SFP(n=20)
	t073	08-16-02-16-13-17-34-16-34	0	1	0
	t230	08-16-02-16-34	0	1	0
	t377	04-02-12-21-17-34-22-25	0	0	1
	t383	08-16-34-13-16-34	0	0	1
	t445	08-16-20-16-34-13-17-34-16-34	0	1	0
	t630	08-16-02-16-34-17-34-16-34	1	0	0
	t950	08-16-34-17-34-16-34	0	1	0
	t1270	09-34-34-34-17-34-16-34	0	0	2
	t1574	08-16-02-16-34-13-13-17-34-16-34	1	0	0
	t4460	08-16-02-16-17-34-16-34	0	1	0
	t5599	08-16-02-16-34-13-16-34-16-34	1	0	0
	t6969	08-16-13-17-34-16-34-34	0	0	1
	t8454	08-16-02-16-34-16-34-13-17-34-16-34	0	1	0
CC50	t246	04-17-23-24-20-17-25	0	1	0
	t8018	04-20-22-17	0	1	0
CC59	t216	04-20-17-20-17-31-16-34	2	6	0
	t270	14-44-13-12-17-17-17-17-23-18-17	1	0	0
	t437	04-20-17-20-17-25-34	0	1	0
CC78	t186[a]	07-12-21-17-13-13-34-34-33-34	0	1	0
	t912	08-12-17-13-13-34-13	0	0	1
	t1814	07-12-21-17-34-34-34-33-34	1	0	0
CC97	t267	07-23-12-21-17-34-34-34-33-34	0	1	0
	t276	15-12-16-02-16-02-25	0	1	0
	t359	07-23-12-21-17-34-34-33-34	1	0	0
CC101	t056	04-20-12-17-20-17-12-17-17	1	1	1
	t2888	04-20-12-17-13-17	0	1	0
CC121	t159	14-44-13-12-17-17-23-18-17	1	0	0
	t272	14-44-13-12-17-17-17-17-23-18-17	0	1	0
	t645	14-44-13-12-17-23-18-17	1	0	0
CC398	t011[b]	08-16-02-25-34-24-25	1	0	0
	t571	08-16-02-25-02-25-34-25	1	0	0

[a] *spa* types comprising *mecA* positive SAI isolates (SAI8: t954/CC5, SAI9: t018/CC30, SAI12: t015/CC45, SAI36: t186/CC78)

[b] *spa* type comprising the *mecA* positive isolate obtained from nasal colonization SANC11

associated with resistance to tetracycline (*tetK/M*) and methicillin (*mecA*). One SANC (SANC11) and four SAI isolates (SAI8, SAI9, SAI12, SAI36) possessed *mecA*. SANC11 was detected in a nasal swab from a female veterinarian aged 27 that could be assigned to ST398-MRSA-V ("Dutch Pig Strain", score: 93.1%). SAI8 was isolated from a skin lesion in a 58-year-old male patient suffering from sepsis. SAI9 was detected in a pharyngeal swab from a 76-year-old male patient and was assigned to ST36/39-MRSA-II, UK-EMRSA-16 (synonym to USA 200, Irish AR7.0, Canadian MRSA-4; score: 94.3%). SAI12 was isolated from a perineal/perianal swab of an 85-year-old male and was assigned to ST45-MRSA-IV, Berlin EMRSA (synonym to USA 600-MRSA-IV, score: 91.8%). SAI 36 was isolated from a 42-year-old female suffering from ulceration after a burn wound and could be assigned to CC78-MRSA-IV, WA MRSA-2 (score: 96.0%).

DNA microarray results for genes encoding superantigenic toxins are displayed in Table 6. We tested for genes encoding staphylococcal enterotoxins (*entA-entJ*), enterotoxin-like proteins (*entK-entR, entU*), as well as exfoliative toxins (*entA/B/D*), toxic shock syndrome toxin (*tst-1*), and panton valentine leukocidin (*pvl*). While *entA-entD* were found in isolates of all three sources, *entE* was not detected. SFP isolates were significantly more likely to possess enterotoxin A variant *entA-320* than SANC (p=0.005) and SAI isolates (p=0.002). In comparison with SFP isolates, SANC isolates exhibited enterotoxin A variant *entA-N315* in significantly higher (p=0.042) and *entD* in significantly lower numbers.

Table 4 Assignment to *agr* and *capsule* types based on DNA microarray analysis. The percentages represent the fragment of *S. aureus* nasal colonization (SANC), clinical cases of infection (SAI) and staphylococcal food poisoning (SFP) isolates, for which genes were determined to be present. Calculations include positive signals only, ambiguous signals were omitted

Group	Gene	Source		
		SANC (n=50)	SAI (n=50)	SFP (n=20)
agr types	*agr*I	36%[a SAI]	70%[a SANC]	55%
	*agr*II	28%	22%	25%
	*agr*III	28%[a SAI]	8%[a SANC]	20%
	*agr*IV	6%	12%	5%
capsule types	*capsule*-1	0%	0%	0%
	capsule-5	28%	26%	25%
	capsule-8	72%	74%	75%

[a] Result for this source differs significantly from the result calculated for one (source indicated) or both other sources of isolates investigated in this study (p<0.05)

We observed an even distribution of genes belonging to the enterotoxin gene cluster (*entG, entI, entM, entN, entO, entU*). SFP isolates were shown to possess *entJ* and *entR* in significantly higher numbers than isolates obtained from

Table 5 Genes involved in antibiotic resistance. Percentages of *S. aureus* nasal colonization (SANC), clinical cases of infection (SAI) and staphylococcal food poisoning (SFP) isolates, for which genes were determined to be present based on DNA microarray analysis. Calculations include positive signals only, ambiguous signals were omitted

Gene	Affected antibiotic	Source		
		SANC(n=50)	SAI(n=50)	SFP(n=20)
mecA	Methicillin	2%	8%	0%
blaZ	Beta-lactam	74%	76%	85%
ermA	Macrolides, lincosamides, streptogramin	8%	6%	0%
ermB	Macrolides, lincosamides, streptogramin	2%	2%	5%
ermC	Macrolides, lincosamides, streptogramin	2%	0%	5%
linA	Lincosamide	0%	2%	0%
mrsA	Macrolides	0%	0%	0%
mefA	Macrolides	0%	0%	0%
mpbBM	Macrolides	0%	0%	0%
vatA	Streptogramin	0%	0%	0%
vatB	Streptogramin	0%	0%	0%
vga, b	Streptogramin	0%	0%	0%
vgaA	Streptogramin	0%	0%	0%
aacA-aphaD	Aaminoglycosides (gentamicin, tobramycin)	2%	2%	0%
aadD	Aminoglycosides (gentamicin, tobramycin)	0%	2%	3%
aphA	Aminoglycosides (gentamicin, tobramycin)	0%	2%	0%
sat	Streptothricin	0%	2%	0%
dfrA	Trimethoprim	0%	4%	0%
far	Fusidic acid	0%	0%	0%
mupR	Mupirocin	0%	0%	0%
tetK	Tetracycline	2%	8%	10%
tetM	Tetracycline	2%	4%	0%
cat	Chloramphenicol	0%	0%	0%
fexA	Chloramphenicol	0%	2%	0%
fosB	Fosfomycin, bleomycin	68%[a SAI]	38%[a SANC]	65%
vanA, Z	Vancomycin	0%	0%	0%
vanB	Vancomycin	0%	0%	0%
qacA, C	Unspecific efflux pump	0%	4%	5%

[a] Result for this source differs significantly from the result calculated for one (source indicated) or both other sources of isolates investigated in this study (p<0.05)

Table 6 Genes encoding superantigenic toxins, such as genes coding for staphylococcal enterotoxins (*entA- entJ*) and enterotoxin-like proteins (*entK-entR, entU*), as well as exfoliative toxins (*etA/B/D)*, toxic shock syndrome toxin (*tst*) and panton valentine leukocidin (*pvl*). Percentages of *S. aureus* nasal colonization (SANC), clinical cases of infection (SAI) and staphylococcal food poisoning (SFP) isolates, for which genes were determined to be present based on DNA microarray analysis. Calculations include positive signals only, ambiguous signals were omitted

Group	Gene	SANC(n=50)	SAI(n=50)	SFP(n=20)
Enterotoxins	entA	26%	20%	30%
	entA-320	6%	4%	30%[a]
	entA-N315	18%[a SFP]	16%	0%[a SANC]
	entB	8%	22%	5%
	entC	16%	26%	20%
	entD	2%[a SFP]	6%	15%[a SANC]
	entE	0%	0%	0%
	entG	58%	44%	50%
	entH	4%	4%	0%
	entI	64%	50%	50%
	entJ	2%[a SFP]	6%	15%[a SANC]
Enterotoxin-like proteins	entK	4%	18%[a]	0%
	entL	16%	26%	20%
	entM	68%	50%	50%
	entN	66%	50%	50%
	entO	64%	50%	45%
	entQ	4%[a SAI]	16%[a SANC]	0%
	entR	2%[a SFP]	6%	15%[a SANC]
	entU	46%	50%	50%
Exfoliative toxins	etA	2%	2%	5%
	etB	2%	0%	0%
	etD	2%	0%	0%
Toxic shock syndrome toxin	tst-1	12%	8%	15%
Panton valentine leukocidin	pvl	0%	2%	6%

[a] Result for this source differs significantly from the result calculated for one (source indicated) or both other sources of isolates investigated in this study (p<0.05)

nasal colonization (p=0.034 each). SAI isolates exhibited significantly higher numbers of *entQ* than SANC isolates (p=0.046) and significantly higher numbers of *entK* than both SANC (p=0.023) and SFP (p=0.040) isolates. Few isolates also possessed *tst-1*, *pvl*, and genes encoding exfoliative toxins, with no significant differences in prevalence among isolates of the three investigated sources.

DNA microarray results for genes encoding leukocidins, hemolysins and staphylokinase are depicted in Table 7. The genes were evenly distributed among isolates of all three

Table 7 Other virulence determinants, including genes encoding leukocidins, hemolysins, and staphylokinase. Percentages represent the fraction of *S. aureus* nasal colonization (SANC), clinical cases of infection (SAI) and staphylococcal food poisoning (SFP) isolates for which the genes were determined to be present based on DNA microarray analysis. Results depicted include positive signals only, ambiguous signals were not considered for the calculation

Gene	Protein	Source		
		SANC(n=50)	SAI(n=50)	SFP(n=20)
lukD	Leukocidin D	34%	54%	50%
lukE	Leukocidin E	28%[a SAI]	56%[a SANC]	35%
lukF	Leukocidin F/ hemolysin gamma (B)[1]	86%	96%	100%
lukS	Leukocidin S/ hemolysin gamma (C)[1]	78%	78%	85%
hla	Alpha toxin/ hemolysin alpha	94%	96%	95%
hlb	Beta toxin/ hemolysin beta	56%	62%	40%
hlgA	Gamma toxin/ hemolysin gamma (A)[1]	68%	100%[a]	80%
hld	Delta toxin/ hemolysin delta	100%	100%	100%
sak	Staphylokinase	72%	80%	60%

[a] Result for this source differs significantly from the result calculated for one (source indicated) or both other sources of isolates investigated in this study (p<0.05)

sources, with the exception of *lukE* and *hlgA*. SAI isolates possessed *lukE* significantly more frequently than SANC isolates (p=0.005), and *hlgA* significantly more often than both SANC (p=0.000) and SFP isolates (p=0.017).

Comparison of microarray profiles using the SplitsTree software resulted in no source-specific clusters, but a mixed distribution of isolates of all three sources (see Fig. 1).

Discussion

Screening nasal swabs for SANC isolates showed a nasal carriage rate of 38%. CC30 and CC45 represented the most common clonal complexes among nasal isolates investigated, comprising 24% and 16% of SANC isolates, respectively. These findings are consistent with a recent study conducted in Switzerland which observed a nasal carriage rate of *S. aureus* of 32% among healthy adults and reported CC30 and CC45 to be the most common clonal complexes among SANC isolates, comprising 24% of nasal carriage isolates each [15]. It was reported that CC30 occurs at high frequencies and is stably maintained among human carriers worldwide [15–17]. A recent study conducted among asymptomatic carriers in Germany found CC8, CC15, CC30, and CC45 to be most common among asymptomatic carriers [18]. Among the tested SAI isolates, CC45 (20%) represented the dominant clonal complex, while a comprehensive Dutch study observed this clonal complex to be underrepresented among invasive strains [19]. A recent German study found CC8 and CC45 to be most common among *S. aureus* isolated from bone and joint infections [20]. The clonal complexes CC5 and CC30 that were also present among SAI isolates in our study. Isolates assigned to these clonal complexes were observed to significantly increase hematogenous complications in staphylococcal infections in humans [21]. Several clonal complexes found in our study among SFP isolates were also present among the investigated SANC and SAI isolates (CC8, CC15, CC30, CC45, CC78, CC101). SFP

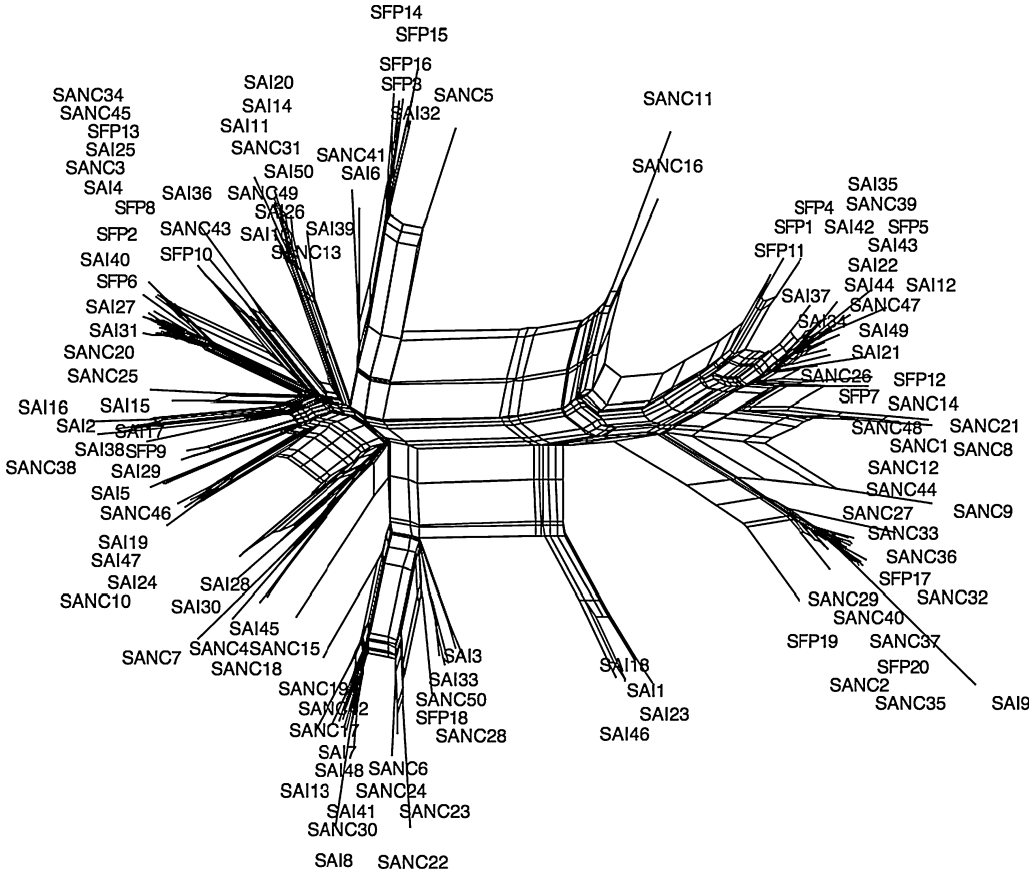

Fig. 1 SplitsTree showing similarity between gene profiles determined by DNA microarray analysis for 120 *S. aureus* isolates, comprising 50 isolates obtained from nasal colonization (SANC), 50 isolates collected from clinical cases of infection (SAI) and 20 isolates associated with staphylococcal food poisoning (SFP)

isolates were most frequently assigned to CC45 (30%), followed by CC8 (20%), CC15 (20%), and CC30 (15%). To the authors knowledge, there have been no previous studies on the distribution of clonal complexes among *S. aureus* isolates associated with outbreaks of SFP.

The *spa* types t008, t015, t018, t056, and t084 that we detected among SFP isolates were also present among SANC and SAI isolates investigated in our study. *Spa* types t008, t015, t056, and t084 were reported among methicillin-sensitive *S. aureus* causing infections in humans [22, 23] and *spa* type t018 was found in common MRSA clones in the United Kingdom and Denmark [24, 25].

DNA microarray profiling enabled the comparison of gene profiles of isolates from nasal colonization, clinical cases of infection and SFP. Interestingly, DNA microarray profiles of isolates from all three sources were rather similar. This is consistent with a recent study that found nasal carriage isolates and clinical isolates to be closely related [26]. Interestingly, especially few significant differences in prevalence rates were found when SFP and SAI isolates were compared.

Among each source of isolates investigated in this study, all *agr* types (*agrI-IV*) were found. The *agrIV* group was recently hypothesized to constitute a truly monophyletic group, while *agrI-III* might have evolved from several unrelated ancestors [10]. DNA microarray results in our study revealed a variety of isolates exhibiting differing virulence gene profiles that possessed *agrIV*. All isolates investigated in our study belonged to capsule type 5 or 8, which were reported to be the only capsular serotypes associated with human disease [27]. The spread of genes conferring resistance to antibiotic agents was corroborated by the antibiotic resistance determinants detected among the *S. aureus* investigated in our study. The most common resistance gene was *blaZ*, encoding penicillinase BlaZ, which enables hydrolysis of both methicillin and oxacillin, was high in isolates from all three sources (SANC: 74%, SAI: 76%, SFP: 85%). The detected prevalence rates for *blaZ* and *mecA* among SANC isolates (*blaZ*: 74%, *mecA*: 2%) are consistent with a recent German report on asymptomatic carriers, which found *blaZ* in 71% and *mecA* in 2% of staphylococcal isolates [18]. A study characterizing *S. aureus* from bone and joint infections detected *blaZ* in 65% and *mecA* in 6% of isolates, similar to the prevalence rates of *blaZ* and *mecA* genes among SAI isolates investigated in this study (*blaZ*: 76%, *mecA*: 8%) [20].

Four out of five *mecA* positive isolates detected in this study were obtained from clinical cases of staphylococcal infection. The MRSA isolate obtained from a nasal swab (SANC11) of a veterinarian working in equine practice belonged to *spa* type t011 and clonal complex CC398, which were also found among clinical MRSA isolates collected from a human patient and several horses in a recent Finnish study [28].

Both tested variants of *entA* encoding enterotoxin A, the gene responsible for most cases of SFP, were detected among SANC and SAI isolates. Interestingly, all SFP isolates possessed the *entA-320* variant, which was first detected in a French field isolate in 2003 [29]. While prevalence rates of *entA* and *entC* detected among SANC isolates in this study were almost identical to those of a study conducted with nasal carriage isolates of restaurant workers in Kuwait city, we found lower prevalence rates of *entB*, *entD*, and *entE* [30]. While a German study reported similar rates of *entB* and *entC* among asymptomatic nasal carriers, slightly lower rates of *entA*, as well as higher rates of *entD* were found [18].

Comparison of microarray profiles using the SplitsTree software resulted in no source-specific clustering, but a mixed distribution of isolates of all three sources. In addition, in our study we found considerable overlap in *spa* types for SFP isolates with isolates collected from nasal colonization and clinical cases of infection. These results suggest contamination of foodstuff during preparation by food handlers that are colonized or infected by *S. aureus* represents a source of SFP.

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4.5 Publication 5

Microarray-based characterization of *Staphylococcus aureus* isolates obtained from chicken carcasses

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Research Note

Microarray-Based Characterization of *Staphylococcus aureus* Isolates Obtained from Chicken Carcasses

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ABSTRACT

A total of 34 *Staphylococcus aureus* isolates from flock-wise pooled chicken neck skin samples collected at two abattoirs during slaughter were characterized with DNA microarray analysis and *spa* typing. The 20 isolates from abattoir A all belonged to clonal complex (CC) 12 and *spa* type t160. Of the 14 isolates from abattoir B, 7 belonged to CC5–t3478, 5 to CC12–t160, 1 to CC45–t040, and 1 to CC101–t056. Of the various resistance-associated genes tested, only *blaZ/R/I* (6 isolates of CC12 and CC101 from abattoir B), *sdrM* ($n = 34$), *fosB* ($n = 33$), and *qacC* ($n = 22$) were detected. None of the isolates harbored genes conferring methicillin resistance. In terms of genes encoding enterotoxins, *seb* (all isolates of CC12), *egc* (*seg, sei, selm, seln, selo, selu*; all isolates of CC5 and CC45), and *sea* (14 isolates of CC12 and 1 isolate of CC5) were found. In addition, all isolates harbored genes for intracellular adhesion proteins (*icaA/C/D*) and were positive for *cap5* or *cap8* (capsule type 5 or 8). Comparison of DNA microarray profiles identified four categories comprising (i) all isolates of CC12, (ii) all isolates of CC5, (iii) the CC45 isolate, and (iv) the CC101 isolate. The high similarity of the isolates from abattoir A could indicate contamination of chicken carcasses with *S. aureus* persisting on the slaughter equipment, but further investigations are required to elucidate potential contamination routes.

In addition to being a commensal colonizer of many animal species and humans, *Staphylococcus aureus* is also involved in a variety of different symptoms and diseases. In poultry, *S. aureus* has been described associated with infections such as septic arthritis, osteomyelitis, subdermal abscesses, and gangrenous dermatitis *(2)*. On the other hand, *S. aureus* can also cause staphylococcal food poisoning in humans, one of the most prevalent foodborne intoxications worldwide. Staphylococcal food poisoning results from ingestion of heat-stable staphylococcal enterotoxins (SEs) preformed in food and is characterized by nausea, emesis, abdominal cramps, and diarrhea *(9)*. In particular, the classical SEs (SEA–SEE) are of major importance in causing staphylococcal food poisoning outbreaks.

There are several reports about the presence of *S. aureus* in domestic and wild birds, but genotyping data of *S. aureus* isolates from healthy chicken or carcasses are widely lacking. Studies reporting characterization data of *S. aureus* from poultry, mainly focused on isolates from diseased animals *(4, 6, 12)*. For typing of *S. aureus,* various methods are established. In addition to *spa* typing, which is frequently used for epidemiological investigations, DNA microarrays facilitating rapid detection of resistance determinants, SE genes, and a variety of virulence genes and typing markers have recently been applied *(11, 20)*. In the present study, 34 *S. aureus* isolates from flock-wise pooled chicken neck skin samples collected at two abattoirs were characterized with DNA microarray analysis. The aim was to determine the gene profiles in order to establish genetic relationships and to compare the isolates from the two abattoirs.

MATERIALS AND METHODS

***S. aureus* isolates.** Thirty-four *S. aureus* isolates obtained from flock-wise pooled chicken neck skin samples collected at two abattoirs (A and B) were investigated in this study. In brief, flock-wise pooled chicken neck skin samples were collected after plucking (abattoir A) and after evisceration (abattoirs A and B) during a 3-month period (September to November 2012). A flock-wise pooled chicken neck skin sample consisted of samples from 10 animals. Overall, 244 (flock-wise pooled) samples were collected. Each of these pooled samples represented a different flock. Of the 191 (flock-wise pooled) samples from abattoir A, 134 were collected after evisceration on 11 sampling days and 57 after plucking on 5 sampling days (10 to 14 samples per day). The 53 (flock-wise pooled) samples from abattoir B were collected on 18 sampling days. Sampled flocks originated from farms throughout Switzerland, and slaughtered animals aged between 30 and 63 days. Of the 34 investigated *S. aureus* isolates (each from a different flock), 20 originating from abattoir A and 14 from abattoir B. *S. aureus* isolates from abattoir A originated from 10 different sampling days and were obtained from samples collected after evisceration and after plucking. Isolates from abattoir B originated from 12 different sampling days.

For isolation of the 34 *S. aureus* isolates, samples were homogenized (30 s) and incubated (24 h, 37°C) in buffered peptone water (Oxoid AG, Pratteln, Switzerland) supplemented with 6.5% NaCl before subsets were plated onto rabbit plasma

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TABLE 1. *Characterization of the 34* Staphylococcus aureus *isolates obtained from herd-wise pooled chicken neck skin samples collected at two abattoirs: assigned clonal complexes,* spa *types, presence of genes encoding resistance, SEs and enterotoxin-like proteins, and capsule- and biofilm-associated markers*

Abattoir	No. of isolates	Clonal complex	*spa* type	Resistance-associated genes[a,b]	Genes encoding enterotoxins and enterotoxin-like proteins[a]	Capsule- and biofilm-associated genes[a]
A	20	CC12	t160	*fosB, qacC, sdrM*	*sea*-N315 (9), *seb,* ORF CM14	*cap8* (*capH8/I8/J8/K8*), *icaA/C/D*
B	7	CC5	t3478	*fosB, qacC* (1), *sdrM*	*sea*-320E (1), *seg/sei/selm/ seln/ selo/selu* (*egc*)	*cap5* (*capH5/J5/K5*), *icaA/C/D*
	5	CC12	t160	*blaZ/I/R, fosB, sdrM*	*sea*-N315, *seb,* ORF CM14	*cap8* (*capH8/I8/J8/K8*), *icaA/C/D*
	1	CC45	t040	*sdrM*	*seg/sei/selm/seln/selo/selu* (*egc*)	*cap8* (*capH8/I8/J8/K8*), *icaA/C/D*
	1	CC101	t056	*blaZ/I/R, fosB, qacC, sdrM*	ND[c]	*cap8* (*capH8/I8/J8/K8*), *icaA/C/D*

[a] When the respective gene was not harbored by all isolates, the number of positive isolates is indicated in parentheses.
[b] Resistance genes not detected for methicillin (*mecA/I/R*), macrolides (*ermA/B/C, msrA, mefA, mphC*), lincosamides (*lnuA, vgaA, cfr*), virginiamycin (*vatA, vgBA*), streptogramin (*vatB, vgaA, cfr*), aminoglycosides (*aacA–aphD*, *aadD*, *aphA3*), streptothricin (*sat*), trimethoprim (*drfS*1), fusidic acid (*far*1), mupirocin (*mupA*), tetracycline (*tetK/M*), chloramphenicol (*cat*), or chloramphenicol-florfenicol (*fexA*).
[c] ND, no respective genes detected.

fibrinogen agar (48 h, 42°C; Oxoid AG). Isolates were confirmed to represent *S. aureus* by using the Alere StaphType DNA microarray system (Alere Technologies GmbH, Jena, Germany), as described below. Of the isolates confirmed as *S. aureus,* 34 were randomly selected for further analysis. All 34 *S. aureus* isolates were tested for growth on chromID MRSA (methicillin-resistant *Staphylococcus aureus*) agar (24 h, 37°C; bioMérieux, Marcy l'Etoile, France) and penicillin MICs of selected isolates were determined with MIC Test Strip Penicillin G (0.016 to 256 μg/ml; Liofilchem s.r.l., Roseto degli Abruzzi, Italy).

DNA microarray analysis. The 34 *S. aureus* isolates were grown on plate count agar (24 h, 37°C; Oxoid AG). DNA isolation was performed with the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Concentrations of nucleic acids were measured with an ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). For DNA microarray profiling, the StaphType ArrayStrip platform was used according to the manufacturer's instructions (Alere Technologies GmbH). This microarray covers over 300 target sequences including *S. aureus* species markers, resistance-associated genes, genes encoding SEs and enterotoxin-like proteins, capsule- and biofilm-associated markers, and a variety of genes encoding, e.g., leukotoxins and hemolysins. Resulting DNA microarray profiles were used to assign isolates to clonal complexes *(13),* and profiles were converted to sequence-like strings for visualization by SplitsTree4 (http://www.splitstree.org), as previously described *(20).*

***spa* typing.** The sequence of the polymorphic X region of the *spa* gene of each isolate was determined as described by Aires-de-Sousa et al. *(1),* with minor modifications *(20).* Sequencing was outsourced (Microsynth, Balgach, Switzerland), and sequences were assigned to *spa* types by using the *spa* server (http://www.spaserver.ridom.de).

RESULTS AND DISCUSSION

Clonal complex and *spa* type. The 34 *S. aureus* isolates originating from flock-wise pooled chicken neck skin samples were assigned to four clonal complexes (CC) and four *spa* types (Table 1). The 20 isolates from abattoir A all belonged to CC12 and *spa* type t160. Of the 14 isolates from abattoir B, 7 belonged to CC5–t3478, 5 to CC12–t160, 1 to CC45–t040, and 1 to CC101–t056. *S. aureus* of CC12 has so far scarcely been described in poultry, whereas CC5 represents a major *S. aureus* lineage in chicken, especially in diseased animals *(4, 6, 12).* This clade probably became established via a human-to-poultry host jump *(10).*

Resistance-associated genes. Of the variety of resistance-associated genes tested in the DNA microarray analysis, only *blaZ/R/I, sdrM, fosB,* and *qacC* were detected among the 34 *S. aureus* isolates from chicken carcasses (Table 1). The *blaZ/R/I* genes conferring resistance to β-lactams were found in the isolates of CC12 and CC101 from abattoir B. However, determination of penicillin MICs showed that the 5 CC12 *blaZ/R/I*-positive isolates were phenotypically sensitive (MIC of 0.047 to 0.064 μg/ml, breakpoint of ≥0.25 μg/ml). The *blaZ* and *blaI* genes were intact and thus were unlikely to have contributed to the penicillin-sensitive phenotype displayed by these isolates. However, there are various SNPs in the *blaR* genes, which on translation lead to a truncated BlaR protein, which could impact BlaR function (data not shown). Moreover, *sdrM* (putative transport protein), *fosB* (metallotiol transferase, a putative fosfomycin-bleomycin resistance gene), and *qacC* (quaternary ammonium compound resistance protein C) were present in 34, 33, and 22 isolates, respectively. But the functional activity, in particular of *fosB,* remains to be verified. The *qacC* gene was thereby found in all isolates from abattoir A but only in 2 isolates from abattoir B. On the other hand, various other resistance-associated genes were not detected among the *S. aureus* isolates investigated in the present study (Table 1). In particular, none harbored genes conferring methicillin resistance or showed growth on chromID MRSA agar. MRSA has been reported in poultry *(15, 17),* but to date seems to occur mainly in low numbers on chicken carcasses *(5, 8, 19).*

Genes encoding enterotoxins and enterotoxin-like proteins. In terms of genes encoding SEs and enterotoxin-

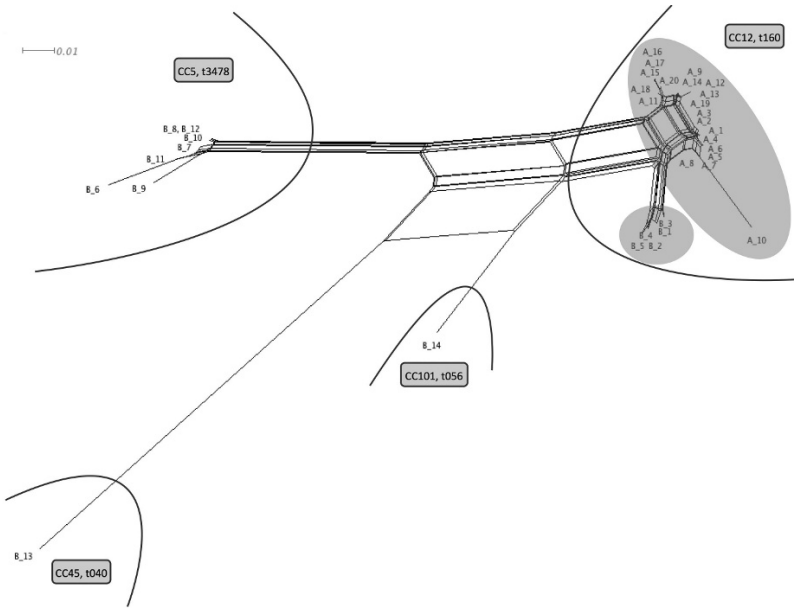

FIGURE 1. *SplitsTree illustrating the similarity among gene profiles gained by DNA microarray analysis for the 34* Staphylococcus aureus *isolates from chicken carcasses (A1 to A20, isolates originating from abattoir A; B1 to B14, isolates originating from abattoir B). Four different categories were identified: (i) isolates of CC12–t160 (A1 to A20, B1 to B5), (ii) isolates of CC5–t3478 (B6 to B12), (iii) the CC45–t040 isolate (B13), and (iv) the CC101–t056 isolate (B14). The predominant CC12–t160 cluster consisted of two subclusters comprising isolates from abattoir A (A1 to A20) and abattoir B (B1 to B5).*

like proteins, DNA microarray analysis identified *sea, seb, egc* (enterotoxin gene cluster: *seg, sei, selm, seln, selo, selu*), and ORF CM14 (enterotoxin-like proteins) among the 34 *S. aureus* isolates from chicken carcasses (Table 1). The 25 CC12 isolates (originating from both abattoirs) were positive for *seb* and ORF CM14, whereas isolates of CC5 and CC45 harbored *egc,* and the CC101 isolate lacked all tested enterotoxin-associated genes. In addition, 14 isolates of CC12 and 1 isolate of CC5 were positive for *sea* (alleles *sea*-N315 or *sea*-320E). Basically, little is known about enterotoxigenic *S. aureus* from chicken. The presence of genes encoding classical SEs in some of our *S. aureus* isolates is remarkable, because genes encoding for SEA–SEE have rarely been described in poultry isolates *(4, 7, 18).* On the other hand, *egc* is commonly found in *S. aureus* from poultry, especially in strains of CC5 *(4, 12, 18).*

Capsule- and biofilm-associated genes. DNA microarray analysis suggests that the CC5 isolates exhibit capsule type 5 and the remaining isolates of CC12, CC45, and CC101 exhibit capsule type 8 (Table 1). Moreover, all 34 *S. aureus* isolates from chicken carcasses harbored genes for intracellular adhesion proteins (*icaA, icaC, icaD*), which play a role in slime production *(3).* In accordance with the results of Nemati et al. *(14),* the gene encoding for the biofilm-associated protein (*bap*) was not detected.

Comparison of DNA microarray profiles. Using the SplitsTree software for comparison of DNA microarray profiles, the 34 *S. aureus* isolates (each from a different flock) could be separated into four different categories comprising closely related isolates: (i) isolates of CC12–t160 ($n = 25$), (ii) isolates of CC5–t3478 ($n = 7$), (iii) the CC45–t040 isolate, and (iv) the CC101–t056 isolate (Fig. 1). Twenty-five *S. aureus* isolates grouped in the CC12–t160 cluster, which consisted of two subclusters. While the first subcluster comprised all isolates from abattoir A, the other subcluster comprised 5 isolates from abattoir B. The high similarity of the *S. aureus* isolates from abattoir A (originating from samples collected after plucking and after evisceration) could indicate contamination of carcasses, with strains persisting on the slaughter equipment. Notermans et al. *(16)* identified plucking and evisceration as main stages for contamination of carcasses with indigenous *S. aureus*. Still, various sources for contamination of carcasses must be considered. Further investigations are therefore required to elucidate contamination routes and to determine whether a certain *S. aureus* population has become established on the processing equipment.

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4.6 Publication 6

Virulence and resistance gene profiles of *Staphylococcus aureus* strains isolated from ready-to-eat foods

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Research Note

Virulence and Resistance Gene Profiles of *Staphylococcus aureus* Strains Isolated from Ready-to-Eat Foods

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ABSTRACT

Staphylococcal food poisoning represents the most prevalent foodborne intoxication worldwide. Oral intake of staphylococcal enterotoxins from food can result in emesis and diarrhea and can be fatal in children and the elderly. Few data have been available on the characteristics and sources of *Staphylococcus aureus* strains isolated from ready-to-eat (RTE) foods. In this study, we used a DNA microarray to determine virulence and antimicrobial resistance gene profiles of *S. aureus* from RTE foods. A total of 267 *S. aureus* strains isolated from 244 RTE foods were investigated. The isolates originated from precooked foods (41% of isolates), meat and fish products (17%), cheese (13%), delicatessen salads (8%), sandwiches and canapés (8%), confectionery and bakery products (6%), and various other RTE foods (7%). Eleven samples (5%), of which 9 were raw milk cheeses, contained $>10^5$ CFU/g, which is considered a health risk. Four *S. aureus* strains were associated with intoxications; three cases were linked to consumption of cheese and one case was linked to consumption of potato salad. DNA microarray results revealed that one-third of the tested strains had at least one major enterotoxin gene (*sea* through *see*). We also detected the toxic shock syndrome gene (18% of isolates) and various genes conferring antimicrobial resistance, including genes involved in resistance to beta-lactams (*blaZ,* 72% of isolates), methicillin (*mecA,* 1% of isolates), and vancomycin (*vanB,* 1% of isolates). *S. aureus* strains were most frequently assigned to clonal complex (CC) 30 (17% of isolates), CC8 (12%), CC15 (11%), and CC45 (10%), which are commonly detected in humans colonized or infected with *S. aureus*. Although a large proportion of the tested food items contained milk, we did not detect CC705, the most prevalent clonal complex among *S. aureus* isolates from bovine mastitis milk. Our results suggest that *S. aureus* isolates from RTE foods do not commonly originate from animals but more likely come from food handlers who contaminate foods.

Staphylococcus aureus can grow in a large spectrum of foods, is the most osmotolerant foodborne pathogen, and is highly resistant to acidic stress *(14)*. *S. aureus* can cause gastrointestinal symptoms such as vomiting and diarrhea through production of staphylococcal enterotoxins (SEs). In contrast to infections with foodborne bacterial pathogens such as *Salmonella,* sporadic cases of staphylococcal food poisoning are not officially reported. However, from 1994 to 2006, coagulase-positive staphylococci were the third most common cause of foodborne outbreaks in Switzerland behind *Campylobacter* and *Salmonella*. Most outbreaks were linked to artisanal cheeses, which are nationally merchandised *(1)*. In the United States, staphylococci account for about 14% of the foodborne outbreaks, and in a single year these infection cause an estimated $1.2 billion in losses *(13)*.

S. aureus infections can cause a wide range of illnesses. With increasing prevalence of *S. aureus* strains resistant to one or several classes of antimicrobial agents, treatment of these infections becomes increasingly difficult. The Centers for Disease Control and Prevention *(2)* therefore categorized methicillin-resistant *S. aureus* (MRSA) as a serious threat and vancomycin-resistant *S. aureus* as a concerning threat. Prevalence of MRSA in pigs, farmers, and veterinarians is comparatively low in Switzerland *(5)*. However, antimicrobial-resistant *S. aureus* strains and genes conferring resistance can be spread from animals to humans by direct contact or through food *(9)*.

Ready-to-eat (RTE) foods are of particular interest because they can be consumed without cooking or other preparation steps. Few data have been available on characteristics and sources of *S. aureus* strains isolated from RTE foods. Currently, the Swiss Ordinance on Hygiene stipulates tolerance values of 100 CFU of coagulase-positive staphylococci per gram of food for the following categories of RTE foods: whipped cream, confectionery and bakery products, raw foods, heat-treated foods, and foods made from mixtures of heat-treated and raw components *(4)*. Values exceeding these tolerance limits indicate that foods were not handled in a hygienic manner and that good manufacturing practices were not used.

In this study, we characterized a collection of *S. aureus* strains from a broad spectrum of RTE foods sampled in

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TABLE 1. *Prevalence rates of* S. aureus *in groups of 244 tested RTE foods*

Food category	Prevalence (%)	Highest contamination	
		Sample	Level (CFU/g)
Cheese	13	Goat cheese	2.6×10^7
Delicatessen salads	8	Potato salad	1.0×10^7
Precooked foods	41	Pasta	2.9×10^5
Different foods	7	Butter from unpasteurized milk	1.3×10^4
Meat and fish products	17	Ham	2.8×10^3
Sandwiches and canapés	8	Chicken and curry sandwich	1.2×10^3
Confectionery, bakery products	6	Mix of apples, mayonnaise, and curd	6.0×10^2

Switzerland to draw epidemiological conclusions concerning origins and reservoirs of strains. This information could be used to derive adequate risk-based preventive measures.

MATERIALS AND METHODS

Collection of staphylococcal isolates. From August 2007 to July 2008, 15 official food control laboratories investigated RTE foods following a modified ISO 6888-2 protocol (spread plate instead of pour plate technique), the official detection method for coagulase-positive staphylococci in Switzerland and the European Union *(7)*. Up to two isolates of coagulase-positive staphylococci per investigated food item were sent to the Swiss Federal Office of Public Health. Isolates were subcultured on Columbia agar with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) for 24 h at 37°C. Isolate identity was confirmed by biochemical testing with API Staph (bioMérieux) and culturing on rabbit plasma fibrinogen agar (Oxoid, Basingstoke, UK) for 24 to 48 h at 37°C. Isolates obtained from the same food item and had identical API codes were considered presumptive duplicates, and one of them was excluded from the study. All other isolates with phenotypes characteristic of *S. aureus* were kept at −70°C until further use.

DNA microarray profiling. DNA microarrays were used to confirm putative *S. aureus* strains and to determine virulence and antimicrobial resistance gene profiles. Probe hybridization patterns allowed assignment of clonal complexes (CCs). The StaphyType ArrayStrip platform (Clondiag Chip Technologies, Jena, Germany) comprising over 300 probes that target antimicrobial resistance and virulence genes was used according to the manufacturer's instructions. Similar to the approach of Coombs et al. *(3)*, microarray profiles were compared using SplitsTree4, software designed to compute unrooted phylogenetic networks from molecular sequence data as previously described *(8, 16)*. Isolates carrying genes involved in methicillin resistance were further analyzed for phenotypical methicillin resistance by streaking them on chromID MRSA test plates (bioMérieux).

RESULTS

The 244 RTE food items in which *S. aureus* had been detected were grouped into seven categories (Table 1). *S. aureus* was most frequently reported in precooked foods (41% of samples) followed by meat and fish products (17%) and cheese (13%). *S. aureus* counts ranged from 10^2 CFU/g in a mix of apples, mayonnaise, and curds to 10^7 CFU/g in an artisanal goat cheese. *S. aureus* counts $>10^5$ CFU/g were detected in cheeses, delicatessen salads, and precooked foods.

Although 46% of the samples had *S. aureus* counts <100 CFU/g and therefore complied with the tolerance range set by the Swiss Ordinance on Hygiene *(4)*, 49% had counts of $>10^2$ to $\leq 10^5$ CFU/g. The limit of 10^5 CFU/g, which is considered a health risk, was exceeded by 11 samples (5%): 9 cheese samples, 1 precooked pasta sample, and 1 potato salad sample (Table 2). Four of these samples (cheese and potato salad) had been associated with cases of intoxication; the potato salad was associated with an outbreak involving 12 persons.

Because more than one *S. aureus* strain was isolated from 14% of the RTE foods, 267 strains were further characterized. One-third of the isolates harbored at least one major enterotoxin gene (*sea* through *see*). The *sea* gene was most frequent, with a prevalence rate of 16% among all

TABLE 2. *Critical counts of* S. aureus *among 244 tested RTE foods*

Food	Count (CFU/g)	Intoxication	Enterotoxin gene(s)
Cheese from goat's milk	2.6×10^7	Yes	*seg, sei*
Soft cheese type Tomme	1.2×10^7	No[a]	ND[b]
Potato salad	1.0×10^7	Yes	*sea, seg, sei*
Fresh cheese	7.6×10^6	Yes	*seg, sei*
Soft cheese type Tomme	8.5×10^5	No	ND
Pasta, precooked	2.9×10^5	No	*sec, seg, sei*
Semihard cheese from goat's and cow's milk	2.6×10^5	No	ND
Soft cheese type Tomme from goat's milk	2.2×10^5	Yes	*sed, sej*
Semihard cheese	1.5×10^5	No	*sea, sed, sej*
Cheese from goat's and cow's milk	1.5×10^5	No	*sec*
Semihard cheese	1.2×10^5	No	*sea, sed, sej*

[a] No cases of food poisoning were reported.
[b] ND, no enterotoxin genes were detected.

TABLE 3. *Prevalence of resistance and virulence genes based on DNA microarray detection among 267* S. aureus *strains isolated from RTE foods*

Gene type	Gene	Function	Prevalence (%)
Resistance[a]	*blaZ/R*	Beta-lactam resistance	72
	blaI	Beta-lactam resistance	75
	mecA	Methicillin resistance	1
	vanB	Vancomycin resistance	1
	ermA/C	Macrolide/clindamycin resistance	1
	msr(A)	Macrolide resistance	2
	aadD	Tobramycin resistance	3
	tet(K)	Tetracycline resistance	5
	tet(M)	Tetracycline resistance	1
	cat	Chloramphenicol resistance gene	1
	fosB	Metallothiol transferase	64
Exfoliative toxins	*etA*	Exfoliative toxin A	1
	etB	Exfoliative toxin B	0
	etD	Exfoliative toxin D	2
Toxic shock syndrome	*tst-1_human*	Toxic shock syndrome (human allele)	17
	tst-1_bovine	Toxic shock syndrome (bovine allele)	1
Capsule	*cap1*	Capsule type 1	0
	cap5	Capsule type 5	39
	cap8	Capsule type 8	61
Accessory gene regulators	*agrI*	Accessory gene regulator type I	57
	agrII	Accessory gene regulator type II	22
	agrIII	Accessory gene regulator type III	21
	agrIV	Accessory gene regulator type IV	0
Others	*lukD/E*	Leukocidin D	57
	lukF	Leukocidin F/hemolysin gamma (B)	98
	lukS	Leukocidin S/hemolysin gamma (C)	78
	hla	Alpha toxin/hemolysin alpha	98
	hld/hlgA	Delta/gamma toxin	100
	sak	Staphylokinase	71

[a] Resistance genes not detected among the tested isolates: *mecI, ermB, lnuA, mefA, mphC, vatA/B, vga/B(A), aacA-aphD, aphA3, sat, dfrS1, far1, mupA, fexA,* and *vanA/Z.*

tested strains, followed by *sec* (11% of strains), *sed* (7%), *seb* (4%), and *see* (1%). Among the newly described enterotoxin genes (*seg* through *sej*), *seg* and *sei* were most frequently detected (*seg,* 50% of strains; *sei*, 49%) followed by *sej* (7%) and *seh* (4%). Selected DNA microarray results regarding resistance genes, various superantigens, leukocidins, hemolysins, and typing markers are listed in Table 3. Two strains had genes involved in methicillin resistance (*mecA* and *delta_mecR*) but were phenotypically methicillin sensitive as determined by streaking them on chromID agar. The *vanB* gene involved in vancomycin resistance was detected in 1% of the strains. A comprehensive overview of all prevalence rates determined by DNA microarray has been previously published *(6).*

Microarray profiling also allowed identification of sequence types and CCs. The most frequent CCs detected among the collection of 267 *S. aureus* strains from RTE foods were CC30 (17% of strains), CC8 (12%), CC15 (11%), and CC45 (10%). CC7 and CC5 were detected in 9% of the strains, CC22 and CC101 in 5% of the strains, and CC1 and CC398 in 3% of the strains. CC9, CC25, and CC97 were detected in only 2% of the strains, and CC6, CC12, CC20, CC50, CC88, CC133, CC182, CC509, ST425, ST1093 were detected in 1% of the strains.

The SplitsTree analysis revealed pronounced variability in virulence and antimicrobial resistance gene profiles among the investigated strains (Fig. 1). Distance between strains in the SplitsTree is correlated with the degree of similarity of virulence and antimicrobial resistance gene patterns; thus, closely related isolates with highly similar gene profiles form clusters. Although strains assigned to the same CC are in general more likely to exhibit similar gene profiles, many CCs detected in our study (CC1, CC5, CC6, CC8, CC9, CC12, CC15, CC25, CC30, CC45, CC97, CC101, CC130, CC133, and CC398) were composed of strains that did not form homogeneous clusters in the SplitsTree and differed markedly in virulence and resistance gene profiles.

DISCUSSION

In our study, *S. aureus* counts of $>10^5$ CFU/g, which are commonly recognized to represent a health risk, were detected in 5% of the samples, predominantly among raw milk cheeses. Four of these samples had been associated with cases of intoxication, including two cases of staphylococcal food poisoning from which only the newly described SE genes *seg* and *sei* have been detected. The relevance of newly described SEs, in particular SEG and

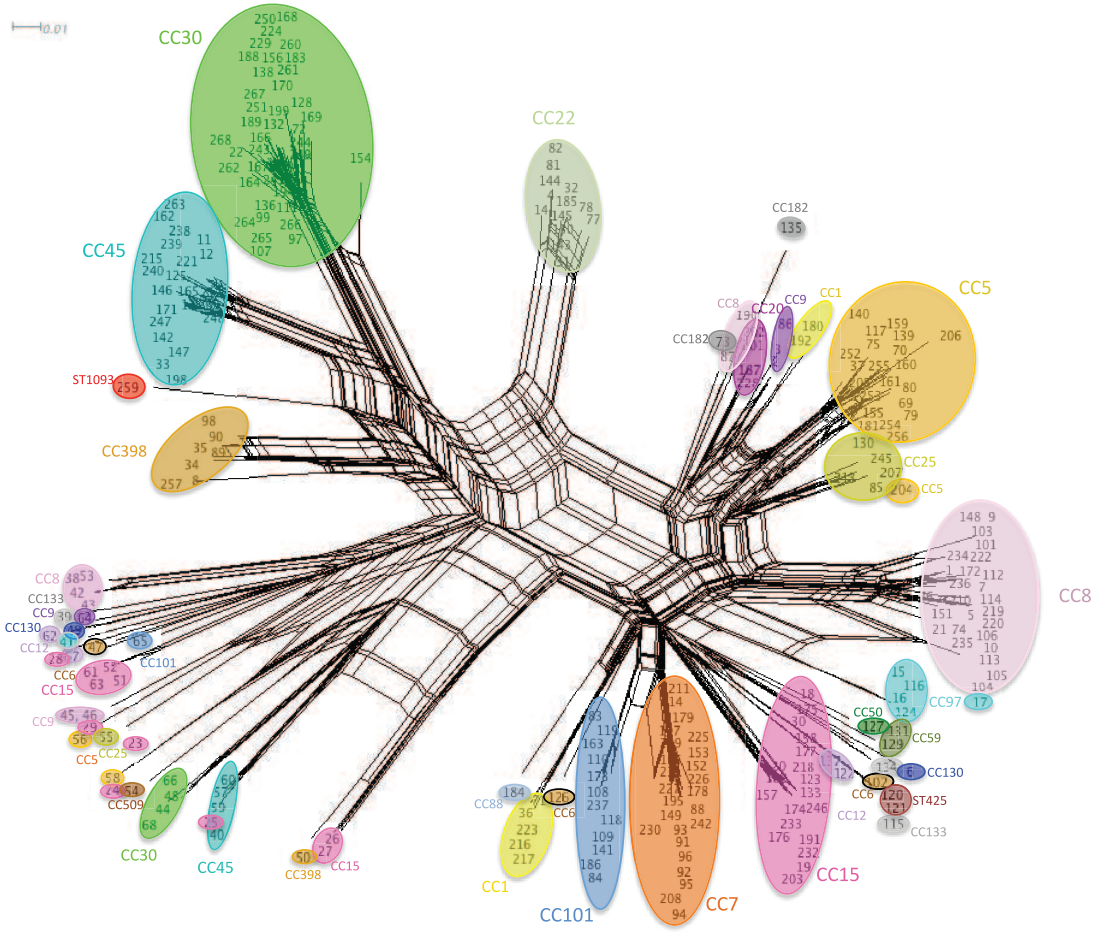

FIGURE 1. *SplitsTree diagram depicting the heterogeneity of virulence and resistance gene profiles detected by DNA microarray analysis among 267* S. aureus *strains isolated from 244 RTE foods. Clusters of strains assigned to the same clonal complex (CC) are depicted as colored circles.*

SEI, has been controversial. SEG and SEI were officially recognized as enterotoxins because they exhibited emetic activity in a monkey feeding assay *(11)*. However, for SEI only weak emetic activity was found *(11),* and no conclusive proof of the relevance of these enterotoxins in humans has been presented. Omoe et al. *(12)* reported that strains harboring *seg* and *sei* produce only very low levels of SEG and SEI. This controversy has been further fueled by the fact that in many outbreaks several SEs have been detected, thus making the identification of the causative SE difficult. The present study is one of only a few studies *(15, 17)* of cases of staphylococcal food poisoning in which only *seg* and *sei* have been detected.

We used a DNA microarray system to determine the prevalence of various virulence and antimicrobial resistance genes among the *S. aureus* isolates from RTE foods. Prevalence rates of genes detected by various probes distinguishing between allelic variants common among *S. aureus* from humans and animals indicated that our strain collection is more likely to originate from human than from animal sources. Although 17% of the tested *S. aureus* strains from RTE foods carried the human allele for toxic shock syndrome, only 1% of the strains had the bovine allele.

The prevalence rates determined for most virulence and antimicrobial resistance genes in this study are highly similar to the prevalence rates of the same genes among *S. aureus* isolates from humans but differ greatly from the prevalence rates detected for these genes among *S. aureus* isolates from animal sources. For instance, 71% of the strains in our study carried *sak,* which encodes staphylokinase. This finding is in accordance with the prevalence rates found for *sak* among *S. aureus* strains isolated from human carriers (72%) and from cases of infections in humans (80%) in Switzerland *(16)*. In contrast, *sak* has been detected among only 12% of *S. aureus* strains isolated from bovine mastitis milk and 3% of *S. aureus* strains isolated from pork in Switzerland *(8)*. A similar pattern was found for *blaZ*. In our study, 72% of the *S. aureus* strains isolated from RTE foods were positive for *blaZ*. Although this rate is very close to the prevalence of *blaZ* among human carriers in Switzerland (74%) *(16)* and Germany (71%) *(10),* it differs greatly from the prevalence of *blaZ* among *S. aureus*

isolates from bovine mastitis milk (24%) and pork (28%). A similar pattern was found in the assignment of strains to CCs. The most prevalent CCs detected in this study can also be found commonly among *S. aureus* strains isolated from people in Switzerland and Germany *(10, 16)* who were colonized or infected with *S. aureus*. In contrast, CC705 (formerly CC151), which is highly prevalent among *S. aureus* isolates from mastitis milk *(8),* was not detected in our strain collection, although various *S. aureus* isolates from milk products were included in the study.

Our results suggest that *S. aureus* strains in RTE foods do not commonly originate from animal sources but most likely are transmitted from symptomatic and/or nonsymptomatic food handlers.

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4.7 Publication 7

Genotyping and DNA microarray based characterization of *Staphylococcus aureus* isolates from rabbit carcasses

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Short communication

Genotyping and DNA microarray based characterization of *Staphylococcus aureus* isolates from rabbit carcasses

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ABSTRACT

Staphylococcus aureus can cause staphylococcal food poisoning. Although the organism is frequently detected on rabbit carcasses, little is known about the characteristics of *S. aureus* strains contaminating rabbit meat. In this study, 137 *S. aureus* isolates originating from 137 rabbit carcasses were *spa* typed and characterized by DNA microarray. The isolates were assigned to CC5, CC7, CC8, CC15, CC96, CC101, CC121, and ST890, and to 13 *spa* types (t056, t085, t091, t160, t179, t681, t741, t745, t1190, t1773, t4770, t8456, t14871). Enterotoxin genes detected included *sea*, *sed*, *sej*, and *ser*. In addition, the *egc* operon, encoding the newly described staphylococcal enterotoxins SEG/SEI/SElM/SElN/SElO/SElU, was found in all isolates except those of t091. While none of the examined isolates presented genes conferring methicillin, vancomycin, or aminoglycoside resistance, we frequently detected *blaZ*/I/R conferring resistance to penicillin. The isolates represented a heterogeneous group assigned to clonal lineages detected among humans and animals, with two *spa* types exclusively associated with rabbit meat (t4770, t8456).

1. Introduction

Rabbit meat has outstanding dietetic and nutritional properties (Dalle Zotte, 2002). The global production of rabbit meat is increasing, with the Food and Agriculture Organization estimating a worldwide yearly production of over 1.7 million tons in 2013 (http://faostat3.fao.org/browse/Q/QA/E).

The contamination of raw meat by staphylococci colonizing the skin of slaughtered livestock represents a major issue for food safety (Rodríguez-Calleja, García-López, García-López, Santos, & Otero, 2006a; Rodríguez-Calleja, García-López, Santos, Otero, & García-Lopez, 2006b). *Staphylococcus* (*S.*) *aureus* is a serious threat to human and animal health and can cause staphylococcal food poisoning (SFP) in humans (Peton & Le Loir, 2014). The latest EFSA report describes an increasing number of SFP outbreaks in the EU, with 386 SFP outbreaks being reported in 2013 (European Food Safety Authority, & European Centre for Disease Prevention and Control, 2015). In previous studies, *S. aureus* has been detected on 30–53% of rabbit carcasses with average cell counts between 1.37 ± 0.79 log CFU/g (Rodríguez-Calleja et al., 2006a; Rodríguez-Calleja et al., 2006b) and 2.05 ± 0.87 log CFU cm^{-2} (Kohler, Krause, Beutin, Stephan, & Zweifel, 2008). However, information about the genomic characteristics and the population structure of *S. aureus* associated with rabbit carcasses is scarce.

DNA sequence-based techniques are the golden standard to determine the genomic population structure of bacteria. Another highly discriminatory rapid and reproducible genotyping method for characterization of *S. aureus* is *spa* typing. This method is based on typing the sequence encoding the polymorphic X region of protein A of *S. aureus*. The X region is constituted by a variable number of repeats and flanked by well-conserved regions.

In this study, we aim to characterize *S. aureus* isolates collected from rabbit carcasses by *spa* typing and DNA microarray analysis in order to assess the population structure and the enterotoxigenic properties of *S. aureus* associated with rabbit meat.

2. Materials and methods

2.1. Isolates and DNA extraction

The 137 *S. aureus* isolates used in this study were isolated from rabbit carcasses in Switzerland (Kohler et al., 2008). *S. aureus* was collected using wet–dry double swabbing of rabbit carcasses and subsequent streaking on Rabbit Plasma Fibrinogen agar (RPF, Oxoid Ltd., Hampshire, UK). Chromosomal DNA of presumptive *S. aureus* was extracted using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) following the manufacturer's instructions.

2.2. spa typing

All 137 *S. aureus* isolates were *spa* typed as previously described (Johler, Layer, & Stephan, 2011) with minor modifications. Briefly, PCR amplification of the polymorphic X region of the *spa* gene was performed and PCR amplicons were purified using the GenElute PCR

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Purification Kit (Sigma-Aldrich, St. Louis, MO, USA). Sequencing was outsourced to Microsynth (Balgach, Switzerland) and assignment to *spa* types was performed using the *spa*-server (http://www.Spaserver.ridom.de/).

2.3. DNA microarray and SplitsTree analysis

A total of 73 isolates were selected for further characterization by DNA microarray. Isolates were selected to reflect maximum diversity regarding sampling date and *spa* types. Of each *spa* type, at least one isolate was further characterized by DNA microarray. DNA microarray was performed using Staphytype genotyping kit 2.0 (Alere, Jena, Germany) following the manufacturer's instructions. An ArrayMate reader (Alere, Jena, Germany) was used for signal acquisition. DNA microarray can be used to determine the presence or absence of over 300 genes and allelic variants, including resistance and virulence genes. It also allows clonal complex (CC) assignment (Monecke, Slickers, & Ehricht, 2008). In addition, the similarity of the virulence and resistance gene profiles was visualized using SplitsTree4 (http://www.splitstree.org/) as previously described (Wattinger, Stephan, Layer, & Johler, 2012).

3. Results

Overall, the *S. aureus* isolates from rabbit carcasses exhibited a high degree of heterogeneity, with SplitsTree analysis revealing three major clusters (Fig. 1). As shown in Table 1, the rabbit meat isolates were assigned to 13 *spa* types (t056, t085, t091, t160, t179, t681, t741, t745, t1190, t1773, t4770, t8456, t14871), seven clonal complexes (CC5, CC7, CC8, CC15, CC96, CC101, CC121) and one singleton (ST890).

Of the 73 isolates, which were characterized by DNA microarray, 66% harbored 2 or more enterotoxin genes, 6% harbored one enterotoxin gene and 26% harbored no enterotoxin genes (Table 1). Moreover, half of the analyzed isolates (37 out of 73) exhibited at least one classical enterotoxin gene (*sea*, *seb*, *sec*, *sed*, *see*). The most frequently detected enterotoxin genes were *seg*, *sei*, *selm*, *seln*, *selo*, and *selu* — genes located in the enterotoxin gene cluster (*egc*). While isolates of the most prevalent *spa* type t8456 exhibited different enterotoxin gene profiles, other *spa* types were exclusively associated with specific enterotoxin genotypes. Isolates assigned to t160 and t179 harbored *sed*, *sej*, and *ser*. Isolates assigned to t091 harbored *sea* only (allelic variant N315).

The presence/absence of further genes encoding superantigens was determined. Two isolates harbored the toxic shock syndrome toxin gene *tst1* (t741 and t745). None of the examined isolates presented the genes encoding for panton valentine leukocidin or the exfoliative toxins *etA*, *etB* or *etD*.

An overview of the detected antimicrobial resistance genes is shown in Table 1. Although all characterized isolates exhibited at least one antimicrobial resistance gene, no isolate presented any genes conferring resistance to methicillin, vancomycin, or aminoglycosides. Genes conferring penicillin resistance (*blaZ*/*I*/*R*) were detected in 25 isolates, which belonged exclusively to t091, t160, t179, and t1773. All isolates assigned to t160 and t179 harbored *sdrM*, *fosB*, and *blaZ*/*I*/*R*.

4. Discussion

While there have been some publications characterizing *S. aureus* isolates from rabbit carcasses and meat (Kohler et al., 2008; Rodríguez-Calleja et al., 2006a; Rodríguez-Calleja et al., 2006b), genomic information about such *S. aureus* remains very scarce. Certain *S. aureus* lineages are predominantly or exclusively associated with specific hosts. In our study we evaluated the population structure of *S. aureus* isolated from rabbit carcasses. The most common CCs found were CC5, CC8, and CC121. *S. aureus* associated with CC5 is commonly detected in human or animal hosts including poultry (Krupa et al., 2014). *S. aureus* associated with CC8 is common among human hosts (Nulens et al., 2008) and cattle (Ruf, Johler, Merz, Stalder, & Hässig, 2015). The only CC in this study that has previously been associated with rabbits is CC121 (Smyth et al., 2009), a CC linked to highly virulent *S. aureus* strains causing infection such as subcutaneous abscesses, pododermatitis and mastitis in rabbits (Vancraeynest et al., 2006).

Of all detected *spa* types in our study, only t741, t745, and t1190 were previously reported in relation to rabbits (Agnoletti et al., 2014;

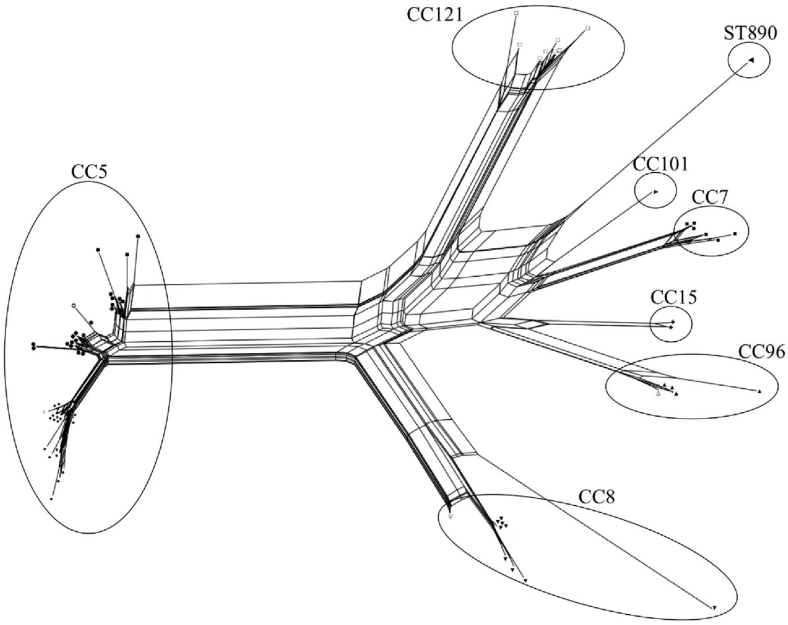

Fig. 1. SplitsTree illustrating similarity of DNA microarray profiles. A total of 73 *S. aureus* isolates obtained from rabbit carcasses were analyzed by DNA microarray. Three major similarity clusters were identified (CC5, CC8, CC121). The predominant CC5 cluster consisted of isolates attributed to t160 (◇), t179 (♦), t8456 (●) and t14871 (○). The CC8 cluster was composed of isolates belonging to t681 (▽) and t4770 (▼), whereas the isolates associated with CC121 belonged solely to t741 (□). The remaining isolates were associated with CC7 (t091 ■), CC15 (t085 ✚), CC96 (t745 ▲ and t1190 △), CC101 (t056 ►) or ST890 (t1773 ◄).

Table 1
Detected clonal complexes (CCs) and singleton (ST), *spa* types, *agr*, capsule types, enterotoxin genes and resistance genes among *S. aureus* isolates from rabbit carcasses. No genes conferring resistance to methicillin, vancomycin, or aminoglycosides were detected. No genes encoding *sec* or *see* were detected.

				Enterotoxin genes								Antimicrobial resistance genes[a]					
CC / ST (n = 73)	*spa* types (n = 137)	*agr*	Capsule	*sea*	*sea* (N315)	*seb*	*sed*	*egc*	*sej*	*ser*	ORF CM14	*blaZ/I/R*	*erm(B)*	*erm(C)*	*tet(K)*	*sdrM*	*fosB*
CC5 (56%)	t160 (1%)	*agrII*	Type 5	1	0	0	1	1	1	1	0	1	0	0	0	1	1
	t179 (24%)			0	0	0	17	17	17	17	0	17	1	0	0	17	17
	t8456 (30%)			2	12	2	0	21	0	2	0	0	0	1	5	21	21
	t14871 (1%)			0	1	0	0	0	0	0	0	0	0	0	0	1	1
CC7 (8%)	t091 (8%)	*agrI*	Type 8	0	6	0	0	0	0	0	0	6	0	0	0	5	0
CC8 (13%)	t681 (1%)	*agrI*	Type 5	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	t4770 (12%)			0	0	0	0	0	0	0	0	0	0	1	0	9	9
CC15 (2%)	t085 (2%)	*agrII*	Type 8	0	0	0	0	0	0	0	0	0	0	0	2	2	2
CC96 (7%)	t745 (6%)	*agrIII*	type 8	0	0	0	0	0	0	0	0	0	1	3	4	4	0
	t1190 (1%)			0	0	0	0	0	0	0	0	0	0	0	1	0	0
CC101 (1%)	t056 (1%)	*agrI*	Type 8	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CC121 (12%)	t741 (12%)	*agrIV*	Type 8	0	0	0	0	8	0	0	8	0	1	2	0	8	8
ST890 (1%)	t1773 (1%)	*agrIV*	Type 8	0	0	0	0	0	0	0	0	1	0	0	0	1	0
			Total (n = 73)	3	19	2	18	47	18	20	8	25	3	7	12	70	61

[a] The detected genes confer resistance to penicillin (*blaZ/I/R*), macrolides/lincosamides (*ermB/C*), tetracycline (*tetK/sdrM*), and fosfomycin (*fosB*).

Vancraeynest et al., 2006), while most other *spa* types were primarily associated with poultry (Krupa et al., 2014), infections or food poisoning in humans (Nulens et al., 2008; Wattinger et al., 2012). The most frequent *spa* types detected in our study were t8456, t179, t741, and t4770. *Spa* types t741 and t745 are associated with high virulence rabbit *S. aureus* (Vancraeynest et al., 2006). The *spa* types t4470 and t8456 have not been reported elsewhere in either rabbit or other hosts. In contrast, t179 was detected among human *S. aureus* isolates (Donker et al., 2009; Espadinha et al., 2013) and was recently found among pigs (Asai et al., 2012). Considering the high prevalence of t4770 and t8456 among the examined rabbit carcass isolates, we hypothesize that *S. aureus* of these *spa* types may be particularly well adapted to the rabbit host.

Overall, the detected prevalence of enterotoxin genes in our study was higher than previously reported (Rodríguez-Calleja et al., 2006a; Rodríguez-Calleja et al., 2006b; Smyth et al., 2005). This may be due to the fact that the DNA microarray covers a wider range of enterotoxin genes and allelic variants. With a prevalence of 64%, the most prevalent finding in our study was the *egc* cluster, which has been associated with outbreaks of SFP (Johler et al., 2015). Concerning the classical staphylococcal enterotoxins, only *sea* (27%), *seb* (3%), and *sed* (25%) have been detected. Interestingly, *sed* was only found among isolates of *spa* types t160 and t179. Isolates assigned to t091 exclusively harbored allelic variant *sea* N315. Among newly described staphylococcal enterotoxins and enterotoxin-like genes, only *ser* and *sej* were found with a prevalence of 27% and 25%, respectively.

As far as antimicrobial resistance profiles were concerned, neither methicillin nor vancomycin nor aminoglycoside resistance genes were detected. Over one third of the examined isolates harbored genes encoding for penicillin resistance (*blaZ/I/R*). The gene *sdrM* encoding for a multidrug efflux pump and the gene *fosB* conferring fosfomycin resistance in interaction with bacillithiol, were highly prevalent among the examined isolates.

All strains harbored *icaA*, *icaC*, and *icaD*. *IcaA* and *icaD* have been associated with slime production (Arciola, Baldassarri, & Montanaro, 2001). Furthermore, these genes play a role in biofilm production (Cramton, Gerke, Schnell, Nichols, & Götz, 1999; Gerke, Kraft, Sussmuth, Schweitzer, & Götz, 1998).

5. Conclusion

The results of our study show that the population structure of the examined rabbit *S. aureus* isolates is highly heterogeneous. While *spa* types t8456 and t4770 were exclusively reported in *S. aureus* from rabbit meat, the majority of isolates was associated with *spa* types and CCs reported in *S. aureus* collected from humans and animal hosts. These findings are of importance to assess *S. aureus* as biological hazard in view of the development of HACCP concepts.

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4.8 Publication 8

Staphylococcus aureus isolates from goat and sheep milk seem to be closely related and differ from isolates detected among bovine milk

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Staphylococcus aureus Isolates from Goat and Sheep Milk Seem to Be Closely Related and Differ from Isolates Detected from Bovine Milk

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Dairy goat and sheep farms suffer severe economic losses due to intramammary infections, with *Staphylococcus aureus* representing the main cause of clinical mastitis in small ruminants. In addition, *S. aureus* contamination of goat and sheep milk may cause staphylococcal food poisoning, as many traditional caprine and ovine milk products are not subjected to pasteurization. Data on virulence and antimicrobial resistance genes, as well as on the clonality of *S. aureus* detected in goat and sheep milk is scarce. Therefore, it was the aim of this study to determine (i) *spa* types and clonal complexes (CC) and (ii) virulence and resistance gene profiles of *S. aureus* isolated from goat and sheep milk. A total of 162 milk samples from sheep and goats presenting signs of an intramammary infection and 104 bulk milk samples were collected. While low prevalence rates of *S. aureus* was detected on single animal level, 46% of the bulk tank milk samples from small ruminants were positive for *S. aureus.* All isolates were *spa* typed and CC and virulence and resistance gene patterns were determined using a DNA microarray. Data from 49 *S. aureus* isolates was included in the statistical analysis and the construction of a SplitsTree. The analyzed isolates could be assigned to eleven CC, with the large majority of goat and sheep isolates being assigned to CC130 and CC133. The findings of this study suggest that *S. aureus* shows pronounced adaptation to small ruminants in general, but not to sheep or goats in particular. Although some common characteristics among *S. aureus* from caprine, ovine, and bovine milk samples were observed, *S. aureus* from small ruminants seem to form a distinct population. As 67% of the detected *S. aureus* strains exhibited at least one enterotoxin gene, many caprine, or ovine raw milk products may be contaminated with low levels of enterotoxigenic *S. aureus*, stressing the importance of strict maintenance of the cold chain.

Keywords: *Staphylococcus aureus*, sheep, goat, clonality, enterotoxin genes, virulence gene profile, mastitis

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INTRODUCTION

Being one of the predominant causes of food poisoning worldwide, *Staphylococcus aureus* is of particular concern to the dairy industry (Oliver et al., 2009). Dairy sheep and goat farms also suffer severe economic losses due to staphylococcal intramammary infections, with *S. aureus* being the main cause of clinical mastitis in small ruminants (Bergonier et al., 2003). However, identification

of affected animals can be challenging, as in contrast to cattle, high somatic cell counts and positive results in the California mastitis test are not necessarily reliable indicators of intramammary infections among small ruminants.

Over the last decade, the production of caprine and ovine milk in Switzerland has been increasing, with 14,000 registered small ruminant farms and a total population of approximately 490,000 heads in 2014 (Swiss Federal Statistical Office). *S. aureus* is one of the most commonly found pathogens in raw caprine and ovine milk (Marogna et al., 2012) and has been detected in over 30% of the examined raw milk of Swiss dairy goat and sheep farms (Muehlherr et al., 2003). As goat and sheep milk are often used for traditional, unpasteurized products such as raw milk cheeses, they represent a potential source of staphylococcal food poisoning (SFP).

The Centers for Disease Control estimate a total number of 240,000 SFP cases per year in the US (Scallan et al., 2011). In the EU, the number of SFP outbreaks is rising, with 386 SFP outbreaks reported in 2014 (Anonymous, 2015). SFP patients present with violent vomiting and diarrhea upon ingestion of staphylococcal enterotoxins pre-formed by *S. aureus* in food (Tranter, 1990). Many different staphylococcal enterotoxins and enterotoxin-like superantigens have been described (Dinges et al., 2000). There is evidence demonstrating emetic activity in humans for all classical enterotoxins SEA-SEE (Dinges et al., 2000) and recently also for some newly described enterotoxins (Jørgensen et al., 2005; Johler et al., 2015).

While the population structure and the genomic characteristics of *S. aureus* from bovine milk are very well described, similar data on *S. aureus* isolated from small ruminants is scarce (Scherrer et al., 2004; Concepción Porrero et al., 2012; Gharsa et al., 2012; Linage et al., 2012; Eriksson et al., 2013; Smith et al., 2014). Data on virulence and antimicrobial resistance genes, as well as on the clonality of *S. aureus* detected in goat and sheep milk is crucial to determine potential routes of transmission, to improve management strategies of affected herds, and to develop effective therapeutic interventions. Therefore, it was the aim of this study to determine clonal complexes (CC) and virulence and resistance gene profiles of *S. aureus* isolated from goat and sheep milk.

MATERIALS AND METHODS

Bacterial Isolation and DNA Extraction

In this study, 162 milk samples of goats ($n = 31$) and sheep ($n = 131$) exhibiting one or several signs of mastitis (increased somatic cell counts, positive California mastitis test, decreased milk yield), as well as 104 raw bulk milk samples were collected from dairy farms in Switzerland (goat farms: $n = 57$; sheep farms: $n = 47$) from March to October 2015. EN ISO 6888-2 was followed for isolation of coagulase-positive staphylococci. One single colony of each different morphology exhibiting an opaque fibrin halo on rabbit plasma fibrinogen agar (Oxoid, Basel, Switzerland) was subcultured. The subcultures were grown on 5% sheep blood agar at 37°C overnight. Chromosomal DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Staphaurex Latex Agglutination Test

All *S. aureus* isolates were subjected to the Staphaurex latex agglutination test (Oxoid, Basel, Switzerland) following the manufacturer's instructions. This assay targets microbial surface components recognizing adhesive matrix molecules (SpA, ClfA, FnBPA, and FnBPB) and frequently yields false-negative results in bovine *S. aureus* (Stutz et al., 2011; Moser et al., 2013).

DNA Microarray, SplitsTree Analysis, and Comparison to Bovine Isolates

DNA microarray analysis was performed using Staphytype genotyping kit 2.0 (Alere, Jena, Germany) following the manufacturer's instructions. The DNA microarray used in this study determines the presence or absence of over 300 different genes and allelic variants, and allows for assignment of CC (Monecke et al., 2008). All presumptive *S. aureus* isolates were further characterized by DNA microarray profiling, which also served as a tool for species confirmation. The DNA microarray hybridization results of isolates from goats and sheep were compared to those of isolates from an unrelated collection of 78 bovine *S. aureus* strains that were obtained in a comprehensive study investigating mastitis isolates from cows in Switzerland (Moser et al., 2013). The resistance and virulence gene profiles of the caprine, ovine, and bovine isolates were visualized using SplitsTree4[1] as previously described (Wattinger et al., 2012).

spa Typing

spa typing, a high resolution single-locus typing technique in *S. aureus*, was performed as previously described (Johler et al., 2011). Briefly, PCR amplicons of the polymorphic X region of the *spa* gene were purified using the GenElute PCR Purification Kit (Sigma–Aldrich, St. Louis, MO, USA), and were subsequently sequenced and assigned to *spa* types[2].

Inclusion Criteria

Stringent inclusion criteria were employed to avoid bias over-representation of strains isolated from both single animals and bulk milk samples of the same dairy farm: only one *S. aureus* isolate was considered for construction of the SplitsTree and statistical analysis, if the analyzed isolates exhibited the same *spa* type and ≤3 different hybridization results in the DNA microarray profiling. Two single animal isolates from sheep were therefore excluded from the study, resulting in 49 *S. aureus* isolates taken into consideration for further analyses.

Statistical Analysis

Statistically significant differences in the distribution of virulence and resistance genes between the bovine, caprine, and ovine isolates were assessed by either Chi squared test or Fisher's exact test (in case $n < 5$) using SPSS 23.0 (IBM Corp., Armonk, NY, USA).

[1] http://www.splitstree.org/

[2] http://www. spaserver.ridom.de/

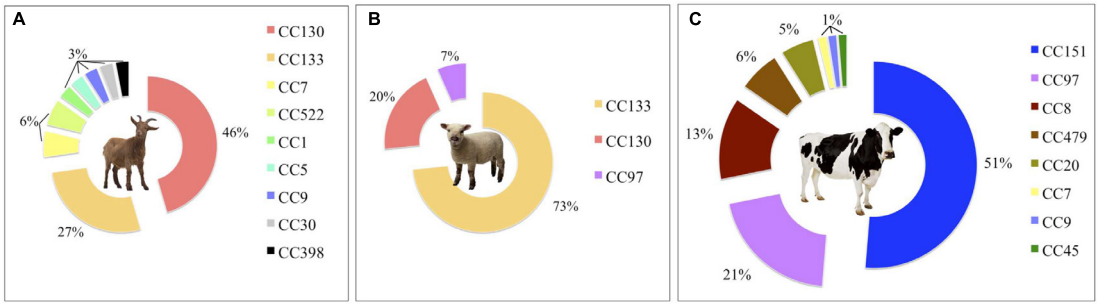

FIGURE 1 | Distribution of clonal complexes (CC) among *Staphylococcus aureus* isolated from the milk of different animal species: (A) goat (*n* = 34, pink), (B) sheep (*n* = 15, blue), (C) cows (*n* = 78, green).

RESULTS

A total of 162 milk samples (goats: $n = 31$; sheep: $n = 131$) of animals presenting signs of an intramammary infection and 104 bulk milk samples (goat farms: $n = 57$; sheep farms: $n = 47$) were collected. On the level of single animals, none of the goat milk samples and 2% ($n = 3$) of the sheep milk samples were positive for *S. aureus*. On the level of bulk milk samples, 60% ($n = 34$) of goat bulk milk samples and 30% ($n = 14$) of sheep bulk milk samples were positive for *S. aureus*, which equals an overall prevalence of 46% among the examined bulk milk samples of small ruminants.

S. aureus from small ruminants were compared to bovine mastitis isolates from the study of Moser et al. (2013), with results being presented in **Figures 1** and **2**, as well as in **Table 1**. The distribution of CC among caprine, ovine, and bovine strains is depicted in **Figure 1**, and a SplitsTree comparing

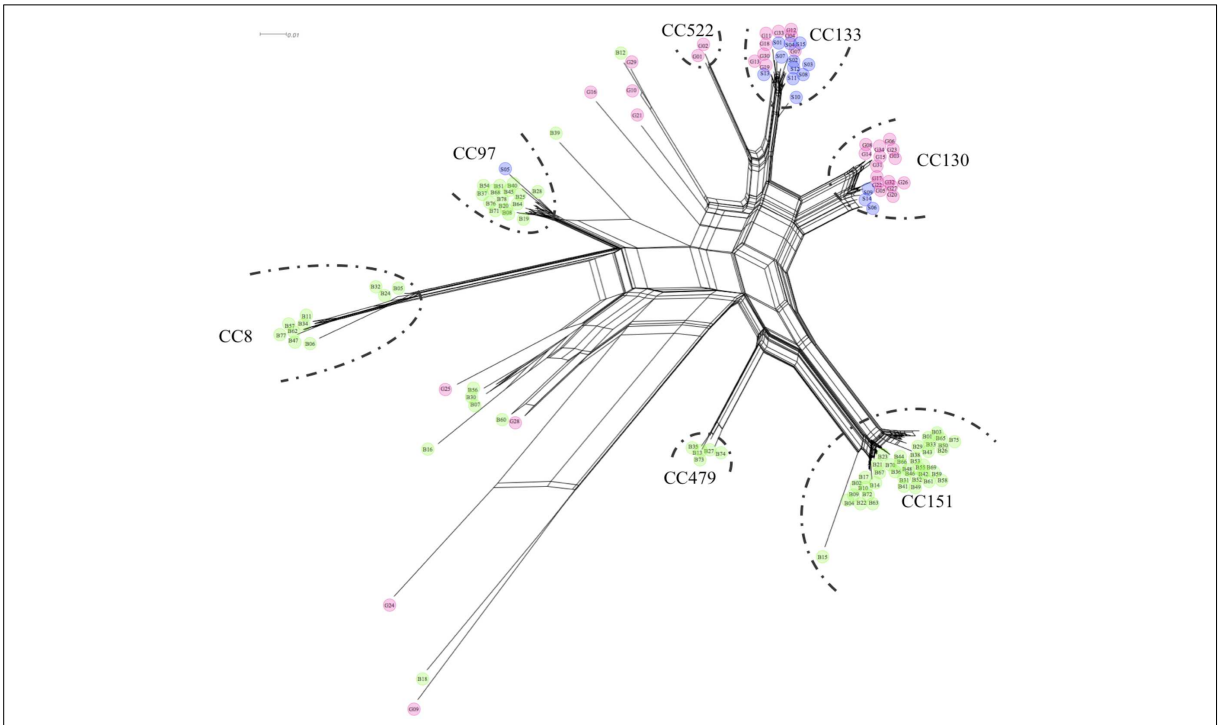

FIGURE 2 | The SplitsTree illustrates similarities between virulence and resistance gene profiles of goat (G), sheep (S), and bovine (B) *S. aureus* isolates. The isolates grouped into clusters mainly based on assignment to CC. Isolates from small ruminants form a distinct population and were mainly found in the clusters of CC130 and CC133. Bovine isolates were predominant in clusters CC8, CC97, CC151, CC479.

TABLE 1 | Prevalence rates of selected virulence and resistance genes detected among *Staphylococcus aureus* strains isolated from goat (G), sheep (S), and bovine (B) milk samples.

Group	Gene/Probe	Function	G ($n = 34$)	S ($n = 15$)	B ($n = 78$)
agr	*agrI*	Accessory gene regulator, type 1	44*^{S}	80*^{G,B}	41*^{S}
	agrII	Accessory gene regulator, type 2	6*^{B}	0*^{B}	59*^{G,S}
	agrIII	Accessory gene regulator, type 3	50*^{S,B}	20*^{G,B}	0*^{G,S}
Capsule	*cap5*	Capsule type 5	9*^{B}	7*^{B}	38*^{G}
	cap8	Capsule type 8	91*^{B}	93*^{B}	62*^{G}
Enterotoxins	*sea*	Enterotoxin A	3	0	10
	sea (320E)	Enterotoxin A, allelic variant 320E	0	0	9
	sea (N315)	Enterotoxin A, allelic variant N315	9	0	1
	seb	Enterotoxin B	0	0	0
	sec	Enterotoxin C	50*^{B}	67*^{B}	15*^{G}
	sed	Enterotoxin D	0*^{B}	0	13*^{G}
	see	Enterotoxin E	0	0	0
	seg	Enterotoxin G	9*^{B}	0*^{B}	65*^{G,S}
	sei	Enterotoxin I	9*^{B}	0*^{B}	65*^{G,S}
	sek	Enterotoxin K	0	0	0
	sel	Enterotoxin L	50*^{B}	67*^{B}	15*^{G,S}
	selm	Enterotoxin-like protein M	9*^{B}	0*^{B}	64*^{G,S}
	seln	Enterotoxin-like protein N	9*^{B}	0*^{B}	65*^{G,S}
	selo	Enterotoxin-like protein O	9*^{B}	0*^{B}	65*^{G,S}
	seq	Enterotoxin Q	0	0	0
	selu	Enterotoxin-like protein U	9*^{B}	0*^{B}	65*^{G,S}
	egc	Enterotoxin Gene Cluster (*seg/sei/selm/seln/selo/selu*)	9*^{B}	0*^{B}	65*^{G,S}
Other superantigens	*tst1* ("bovine" allele)	Toxic Shock Syndrome Toxin, allele from strain RF122	35*^{B}	40*^{B}	8*^{G,S}
	etA/B/D	Exfoliative toxins A, B, and D	0	0	0
	pvl	Panton Valentine Leukocidin	0	0	0
Resistance[1]	*tetK*	Tetracycline	12*^{B}	0	0*^{G}
	fosB	Metallothiol Transferase	38*^{S}	73*^{G}	19
Misc	*sdrD*	Sialoprotein-binding protein D	97*^{B}	100*^{B}	37*^{G,S}
	splE	Serine protease E	59*^{S,B}	27*^{G}	35*^{G}
	lukM/lukF-PV (P83)	Bovine leukocidin	21*^{S,B}	93*^{G}	72*^{G}
	Q7A4X2	Hypothetical protein	47*^{S,B}	80*^{G}	86*^{G}
	ssl06/set21	Staphylococcal superantigen-like protein 6	41*^{S,B}	73*^{G,B}	21*^{G,S}
	ssl10/set4	Staphylococcal superantigen-like protein 10	68*^{S}	93*^{G}	73

A comprehensive list of the prevalence of all genes detected by DNA microarray is provided as a supplement. [1]*None of the isolates harbored the resistance genes mecA, lnuA, msrA, mefA, mphC, vatA/B, vgaA/B, aacA-aphD, aadD, aphA3, sat, dfrS1, far1, Q6GD50, mupA, cat, fexA, cfr, vanA/Z, qacA/C.* **The distribution of the respective gene differed significantly between strains from the stated sources (G, goat, S, sheep, B, bovine) with $p \leq 0.05$.*

gene profiles of caprine, ovine and bovine strains is shown in **Figure 2**. In general, bovine isolates and isolates from small ruminants represent distinct populations, with CC130 and CC133 exclusively associated with small ruminants. Six main SplitsTree clusters, corresponding to CC CC8, CC97, CC130, CC133, CC151, and CC479, were identified. Isolates not associated with one of the main SplitsTree clusters were assigned to CC1, CC5, CC7, CC9, CC30, CC101, and CC398.

An overview of all CC and *spa* types detected is provided in **Table 2**. The isolates analyzed could be assigned to eleven different CC, of which only two (CC130 and CC133) were common among both caprine (71%) and ovine (93%) isolates. A total of 22 different *spa* types were detected. The most prevalent *spa* types were t1773 among the caprine and t1166 among the ovine *S. aureus* isolates, to which 26 and 27% of the analyzed isolates could be assigned, respectively. Three new *spa* types were detected: t15248, t15249, and t15404. While 51% of the bovine *S. aureus* isolates led to false-negative results in the Staphaurex latex agglutination test, all isolates from the milk of small ruminants tested in this study yielded positive results and were thus correctly identified as *S. aureus* by the Staphaurex latex agglutination test.

An overview of the prevalence of the most important virulence and resistance genes detected by DNA microarray is provided in **Table 1**. The supplementary files include a comprehensive list of the prevalence rates of all genes detected (Supplementary Table S1) and a complete overview of all hybridization results (Supplementary Table S2). Overall, 67% of all isolates harbored at least one enterotoxin gene. The most prevalent enterotoxin genes were *sec* and *sel*, which were present in 55% of the isolates from small ruminants. The *sea* gene was found exclusively among caprine isolates. None of the genes encoding exfoliative toxins

TABLE 2 | Clonal complexes (CC) and *spa* types of the *S. aureus* isolates from goat and sheep milk.

Origin	CC	*n*	*spa* type (*n*)	Isolate ID
Goat (*n* = 34)	CC1	1	t127 (1)	G16
	CC5	1	t002 (1)	G25
	CC7	2	t091 (2)	G10, G29
	CC9	1	t899 (1)	G28
	CC30	1	t012 (1)	G09
	CC101	1	t056 (1)	G21
	CC130	15	t1773 (9)	G03, G05, G14, G17, G20, G22, G26, G27, G32
			t11826 (2)	G08, G15
			t15248 (4)	G06, G23, G31, G34
	CC133	9	t544 (1)	G18
			t1166 (2)	G12, G19
			t2678 (3)	G04, G07, G33
			t3583 (1)	G11
			t4735 (1)	G13
			t15249 (1)	G30
	CC398	1	t4475 (1)	G24
	CC522	2	t1534 (1)	G02
			t5428 (1)	G01
Sheep (*n* = 15)	CC97	1	t267 (1)	S05
	CC130	3	t1773 (1)	S09
			t11826 (1)	S14
			t15404 (1)	S06
	CC133	11	t998 (1)	S04
			t1166 (4)	S07, S08, S11, S15
			t2678 (2)	S10, S12
			t3583 (1)	S01
			t4735 (2)	S02, S03
			t6060 (1)	S13

or Panton–Valentine leukocidin were detected. Virulence genes associated with the toxic shock syndrome were found in 27 isolates.

Seven isolates harbored genes conferring penicillin resistance (*blaZ/I/R*). Genes conferring tetracycline resistance were found only among the caprine isolates. All isolates harbored *sdrM*, which encodes a multidrug efflux pump. None of the caprine and ovine isolates harbored genes conferring resistance to methicillin, aminoglycosides, streptogramin A, virginiamycin A, glycopeptides, and vancomycin.

DISCUSSION

The prevalence of *S. aureus* in caprine and ovine bulk tank milk samples varies depending of the country. Muehlherr et al. (2003) detected *S. aureus* in 32% of the caprine and 33% of ovine bulk tank milk samples in Switzerland, while Linage et al. (2012) and Álvarez-Suárez et al. (2015) detected coagulase positive staphylococci in 66% of caprine and 15% of ovine bulk tank milk samples in Spain. Considering the very low prevalence of *S. aureus* detected among the analyzed milk samples of single animals in this study, the overall detected prevalence of *S. aureus* in the bulk milk samples examined was high. This suggests that the prevalence of *S. aureus* as a subclinical agent of mastitis in small ruminant herds in Switzerland may have been underestimated. This is of particular relevance, as SFP has been associated with raw milk from small ruminants (Giezendanner et al., 2009) and as traditional goat and sheep raw milk cheeses are popular.

Most of the isolates characterized in this study were assigned to CC130 and CC133, suggesting that these lineages may represent the major CC among caprine and ovine *S. aureus* isolates in Switzerland. These results are consistent with the findings of previous studies suggesting that predominance of either CC130/CC133 or CC522 in *S. aureus* isolated from milk of small ruminants is associated with geographical, breed- and infection-related aspects (Concepción Porrero et al., 2012; Eriksson et al., 2013; Shepheard et al., 2013; Smith et al., 2014). Only few CC (CC7, CC9, CC97) were detected among strains of both small ruminants and cows.

Even though *S. aureus* isolates originating from caprine and ovine hosts have been *spa* typed in several recent studies (Concepción Porrero et al., 2012; Eriksson et al., 2013; Smith et al., 2014; Bar-Gal et al., 2015), three new *spa* types were detected among the isolates in this study. This suggests that to date, data on the population structure of *S. aureus* isolates originating from small ruminants is still very limited. The *agr* types and *cap* genes detected in this study are consistent with the findings of previous studies investigating *S. aureus* from small ruminants (Alves et al., 2009; Vautor et al., 2009; Bar-Gal et al., 2015).

Most of the isolates analyzed from small ruminants in this study were lacking antibiotic resistance genes. Resistance gene profiles from caprine and ovine strains in this study were not significantly different from those of bovine isolates (Moser et al., 2013). Only the presence of *tetK* in 12% of the caprine isolates was significantly higher compared to ovine and bovine isolates ($p = 0.007$). Overall, the prevalence of *blaZ/I/R* (14%), *tetK* (8%), *tetM* (2%), *ermA/B/C* (2%) detected was lower than the prevalence detected when analyzing *S. aureus* from small ruminants milk or nasal swabs in recent studies from the Middle-East and Africa (Gharsa et al., 2012; Bar-Gal et al., 2015; Jamali et al., 2015). The prevalence of antibiotic resistance genes detected was surprisingly high, considering that herd management differs vastly in small ruminants and cattle, with culling being preferred to antimicrobial treatment in small ruminants.

All isolates harboring *tst1* also harbored the genes *sec* and *sel*, and were assigned exclusively to CC130 and CC133. These genes are located on the ovine pathogenicity island *SaPIov1* (Guinane et al., 2010), and have been previously reported in isolates originating from small ruminants (Smyth et al., 2005; Gharsa et al., 2012). Consistent with findings among *S. aureus* from sheep and goats in Israel (Bar-Gal et al., 2015), in this study, the prevalence of *tst1*, *sec,* and *sel* was significantly higher among small ruminant isolates than among bovine isolates ($p < 0.003$),

which in contrast are more likely to harbor *egc* genes ($p = 0.000$). In this study, the detected overall prevalence of 67% of *S. aureus* carrying at least one enterotoxin gene was similar to 65% reported by Scherrer et al. (2004).

Many genes encoding virulence factors were present at similar rates in caprine, ovine and bovine isolates. This included genes encoding hemolysins (*hla*, *hlb*, *hld*), adhesion factors (*clfA*, *clfB*, *ebps*, *fib*, *fnbA*, *vwb*), hyaluronate lyase (*hysA1/A2)*, immunodominant antigen B (*isaB*), transferrin binding protein (*isdA*) and serine proteases (*splA*, *sspa*). In several studies, these virulence factors have been reported to play a role in mastitis in cattle (Viana et al., 2010; Ote et al., 2011; Wolf et al., 2011). While many genes were equally distributed among small ruminant and bovine isolates, statistically higher prevalence rates of *cap8*, *sdrD*, *sec*, *sel*, *tst1*, *ssl06*, *edinB*, and *Imrp* (*RF122*) among *S. aureus* from small ruminants were observed. As for genes associated with biofilm formation (*icaA/C/D*), very high prevalence rates have been previously reported in isolates originating from small ruminants in particular (Bar-Gal et al., 2015) and from ruminants in general (Snel et al., 2014; Prenafeta et al., 2014).

Comparison of goat and sheep isolates tested in this study showed that caprine and ovine *S. aureus* exhibited highly similar virulence and resistance gene patterns. However, some species-specific patterns were observed. Higher prevalence rates of *splE* among the caprine ($p = 0.038$) and of *lukM* ($p = 0.000$) among the ovine isolates was observed. Simultaneous presence of *splE* and *sdrD*, which was detected in four ovine and 19 caprine isolates in this study, has been associated with gangrenous mastitis in small ruminants (Vautor et al., 2009). In contrast, *lukM* was associated with high leukotoxic activity against bovine polymorphonuclear leukocytes (Rainard et al., 2003) and was hypothesized to play a central role in mastitis in ruminants (Barrio et al., 2006). In addition, significant differences in the prevalence of genes *ssl06/set21*, *ssl10/set4,* and *Q7A4X2* in caprine compared to ovine isolates were observed. While genes encoding for superantigen-like proteins (*ssl*), have been associated with immunoevasion by interfering with the toll-like receptor system (Zecconi and Scali, 2013), Q7A4X2 may be involved in biofilm formation (Snel et al., 2014). These findings suggest that the virulence genes detected, and especially *lukM*, *sdrD,* and *splE,* represent important virulence factors for *S. aureus* strains causing mastitis in small ruminants.

Finally, the performance of the Staphaurex latex agglutination test for identification of *S. aureus* from small ruminants was assessed, as this test was reported to yield false-negative results in 51% of all bovine *S. aureus* strains tested (Moser et al., 2013). The results of this study show that the Staphaurex latex agglutination test system is a highly reliable diagnostic tool for identification of *S. aureus* isolates from caprine and ovine milk samples.

CONCLUSION

The findings of this study suggest that *S. aureus* shows pronounced adaptation to small ruminants in general, but not to sheep or goats in particular. Comparing *S. aureus* from caprine, ovine and bovine milk samples collected in the same country, some common virulence genes were observed, but the results indicate that *S. aureus* from small ruminants may form a distinct population. Further studies covering an extensive strain collection of *S. aureus* from small ruminants collected at various geographical locations are needed to ensure that this finding can be extrapolated to *S. aureus* in general. Although low prevalence rates of *S. aureus* on the level of single animals exhibiting signs of mastitis was detected, 46% of the bulk tank milk samples from small ruminants were positive for *S. aureus.* This suggests that *S. aureus* may pose problems for animal and consumer health, in particular, as many products made from the milk of small ruminants are consumed raw.

AUTHOR CONTRIBUTIONS

SJ and RS conceived and designed the study. AM carried out the laboratory work. AM and SJ analyzed and interpreted the data. AM and SJ wrote the manuscript. All authors critically revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00319

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5 Enterotoxin expression under stress conditions encountered in the food matrix

5.1 Publication 9

Validation of reference genes for normalization of qPCR mRNA expression levels in *Staphylococcus aureus* exposed to osmotic and lactic acid stress conditions encountered during food production and preservation

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RESEARCH LETTER

Validation of reference genes for normalization of qPCR mRNA expression levels in *Staphylococcus aureus* exposed to osmotic and lactic acid stress conditions encountered during food production and preservation

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Staphylococcus aureus; quantitative RT-PCR; reference gene; gene expression; stress adaptation.

Abstract

Staphylococcus aureus represents the most prevalent cause of food-borne intoxications worldwide. While being repressed by competing bacteria in most matrices, this pathogen exhibits crucial competitive advantages during growth at high salt concentrations or low pH, conditions frequently encountered in food production and preservation. We aimed to identify reference genes that could be used to normalize qPCR mRNA expression levels during growth of *S. aureus* in food-related osmotic (NaCl) and acidic (lactic acid) stress adaptation models. Expression stability of nine housekeeping genes was evaluated in full (LB) and nutrient-deficient (CYGP w/o glucose) medium under conditions of osmotic (4.5% NaCl) and acidic stress (lactic acid, pH 6.0) after 2-h exposure. Among the set of candidate reference genes investigated, *rplD*, *rpoB, gyrB*, and *rho* were most stably expressed in LB and thus represent the most suitable reference genes for normalization of qPCR data in osmotic or lactic acid stress models in a rich medium. Under nutrient-deficient conditions, expression of *rho* and *rpoB* was highly stable across all tested conditions. The presented comprehensive data on changes in expression of various *S. aureus* housekeeping genes under conditions of osmotic and lactic acid stress facilitate selection of reference genes for qPCR-based stress response models.

Introduction

Staphylococcal food poisoning is the most prevalent cause of food-borne intoxications worldwide. Consumption of staphylococcal enterotoxins preformed in food causes acute gastroenteritis and can be fatal in sensitive populations such as children and the elderly. *Staphylococcus aureus* represents the most osmotolerant food-borne pathogen and is also highly resistant to acidic stress (Shebuski *et al.*, 2000). Although growth of this organism is repressed by competing bacteria in most matrices, it possesses a crucial competitive advantage under osmotic or acidic stress, conditions that are frequently encountered in food production and preservation (Scott, 1953; Minor & Marth, 1972; Hurst, 1973).

Numerous publications investigated the production of enterotoxins under stress conditions using immunological methods (Genigeorgis & Sadler, 1966; McLean *et al.*, 1968; Troller, 1971; Domenech *et al.*, 1992). However, these data are of limited value, as it was later shown that loss of serological recognition does not equal loss of biological/emetic activity (Bennett, 2005). Quantitative real-time PCR (qPCR) techniques have opened up new possibilities to investigate enterotoxin gene expression under stress conditions. Evaluation of enterotoxin gene expression on mRNA level was suggested to be a useful tool to determine the risk of *S. aureus* intoxication (Lee *et al.*, 2007). However, sound normalization of quantification data is crucial to yield reliable qPCR results. Normalization allows for comparison of mRNA concentrations among different samples, as it controls for variations regarding the yield of both RNA extraction and reverse transcription, as well as amplification efficiency (Bustin *et al.*, 2009). It is widely regarded to be the most appropriate strategy to normalize quantification results using expression levels of several stably expressed house-

keeping genes (HKGs) as an internal reference (Bustin *et al.*, 2009). While qPCR is increasingly used to determine the expression of different enterotoxin genes (Derzelle *et al.*, 2009; Duquenne *et al.*, 2010), there is no information on HKGs suitable for normalization of quantification data of enterotoxin gene expression under osmotic and acidic stress.

In this study, the suitability of nine *S. aureus* HKGs as reference genes for normalization of qPCR mRNA expression levels in food-related stress adaptation models was investigated. We aimed to identify reference genes that could be applied in experimental models investigating enterotoxin gene expression changes related to adaptation of *S. aureus* to NaCl and lactic acid stress encountered during food production and preservation.

Materials and methods

Bacterial strains

Staphylococcus aureus strains used in this study, including sources, clonal complexes, and *spa* types, are listed in Table 1. Strains from different clonal complexes and sources were selected to reflect the variability of *S. aureus* strains that can be detected in foodstuff.

Bacterial growth and collection of samples

Staphylococcus aureus strains were grown and subjected to control (media alone), as well as acid (0.08% lactic acid, pH 6.0) and osmotic (4.5% NaCl) stress conditions in both the nutrient-deficient glucose-free casamino acids yeast extract glycerophosphate broth (CYGP w/o glucose, Novick 1991) and the nutrient-rich medium LB (Bertani, 1951). Media ingredients were obtained from Difco laboratories (Detroit, MI), Oxoid (Cambridge, UK), Becton Dickinson (Allschwil, Switzerland), and Sigma (Buchs, Switzerland). Frozen stock cultures (−80 °C) of *S. aureus* strains were resuscitated by plating on 5% sheep blood agar and incubation at 37 °C over night. Two single colonies per strain were grown in parallel in 10 mL of LB broth cultures for 18 h (37 °C, 225 r.p.m. shaking) to reach the stationary phase. Three identical 5-mL cultures were generated by three times pooling 2.5-mL aliquots of both stationary phase cultures. The pooled cultures were centrifuged (4000 ***g*** for 5 min), and the recovered cells were resuspended in 5 mL of fresh medium adjusted to three different conditions: (1) LB or CYGP only (serving as a control), (2) LB or CYGP w/o glucose adjusted to 4.5% NaCl, (3) LB or CYGP w/o glucose adjusted to pH 6.0 using lactic acid. All samples were incubated in a shaking incubator at 37 °C and 225 r.p.m. for 2 h to allow for adaptation to control and stress conditions. Two samples (400 μL) were taken from each culture, and stress-adapted cells were harvested by centrifugation (8000 ***g***, 5 min, 4 °C). The cells were resuspended in 500 μL RNA protect bacteria reagent (Qiagen, Hombrechtikon, Switzerland), incubated at room temperature for 5 min, and harvested again by centrifugation (3100 ***g***, 5–10 min). Cell pellets were stored at −80 °C. This procedure was repeated to gain two independent samples of each strain grown adapted to these different growth conditions.

Selection of reference genes

Expression stability was analyzed for nine candidate reference genes that have previously been used as references in qPCR studies (Lee *et al.*, 2007; Theis *et al.*, 2007; Derzelle *et al.*, 2009; Duquenne *et al.*, 2010; Stutz *et al.*, 2011). For a detailed list of all selected reference genes and their function see Table 2.

Cell lysis and RNA extraction

Cell pellets were resuspended in 500 μL RLT plus buffer (Qiagen) and mechanically disrupted using a MagNA lyser (Roche, Rotkreuz, Switzerland) in two intervals of 6500 r.p.m. for 60 s with an intermediate cooling step (cells were put on ice for 1 min). Total RNA was isolated using the RNeasy plus mini kit (Qiagen) following the manufacturer's instructions. Two DNA contamination

Table 1. Clonal complexes, *spa* types, and sources of the *Staphylococcus aureus* strains used in this study

Strain ID	Clonal complex	*spa* type	Enterotoxins	Source
KLT_6	CC12	t160	*seb*	Food-borne outbreak
RKI2	CC8	t008	*sea, sed, sej, ser*	Food-borne outbreak
RKI3	CC30	t018	*sea, egc cluster*	Food-borne outbreak
RKI4	CC9	t733	*seb, egc cluster*	Food-borne outbreak
SAI_06	CC97	t276	–	Human bronchial secretion
SAI_23	CC22	t8019	*egc cluster*	Human bronchial secretion
SAK_09	CC5	t8456	*seb, seg, sei*	Rabbit carcass
SAR_1	CC151 (CC705)	t529	*egc cluster*	Bovine mastitis milk

Table 2. Primer pairs, including amplicon sizes, primer concentrations, and *E*-values for the nine HKG in this study

Gene	Function	Primer pair (5′-3′)	Amplicon size (bp)	*E* (%)	Primer c (nM)	Reference
16S rRNA	16S ribosomal RNA subunit	TGT CGT GAG ATG TTG GG CGA TTC CAG CTT CAT GT	270	95	500 500	Stutz *et al.* (2011)
ftsZ	Cell division protein	TAT TAC TGG TGG CGA GTC A AGT ATT TAC GCT TGT TCG GA	223	93	250 250	This study
gyrB	DNA gyrase (subunit B)	GTC GAA GGG GAC TCT G GCT CCA TCC ACA TCG G	242	95	250 250	This study
proC	Pyrroline-5-carboxylate reductase	GGC AGG TAT TCC GAT TG CTT CCG GTG ATA GCT GTT A	231	97	800 1000	This study
pyk	Pyruvate kinase	GCT AGT GAC GTT GCC A ATA GTA CGT GCC GTT G	284	95	500 500	This study
recA	Recombinase A	AAG TAC GTC GTG CAG A TGA CCC ATT CGT TCG C	229	93	250 250	This study
rho	Transcription termination factor	GAA GCT GCT GAA GTC G CGT CCA TAC GTG AAC CC	319	95	250 250	This study
rplD	Ribosomal protein L4	TTC GGA CCA ACT CCA AGA CGA GCA CCT CCT CAA C	352	91	250 250	This study
rpoB	RNA polymerase (beta chain)	CTA AGC ACA GAG GTC GT ACG GCA TCC TCA TAG T	298	98	250 250	This study

removal steps were included by passing the cell lysates through a genomic DNA-binding column and performing an on-column DNase I digestion of the samples bound to the RNA spin column. The RNA templates were eluted in 50 μL RNase-free water. The quantity and quality of RNA were assessed using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and Bioanalyzer (Agilent Technologies, Waldbronn, Germany) instruments, respectively.

Reverse transcription

Reverse transcription was performed using the Quantitect Reverse Transcription Kit (Qiagen). cDNA synthesis was primed using an optimized blend of oligo-dT and random hexamer primers provided in this kit. We converted 480 ng of total RNA of each sample to cDNA in a total volume of 20 μL. We created RT minus controls of each sample by performing the same reaction without reverse transcriptase to be able to subsequently screen each sample for residual DNA contamination in the gene-specific qPCR assay.

Real-time PCR

Primers used are listed in Table 2. Real-time PCR experiments were performed using Light Cycler 480 (Roche). Reactions were performed in a total volume of 10 μL, including 4.8 ng cDNA template (except for 16S rRNA gene primers, for which 0.48 ng cDNA was used), optimized primer concentrations (Table 2), and the LIGHTCYCLER 480 SYBR Green I master mix (Roche). Water (no template) and RT minus samples served as controls. PCR cycling conditions included 8 min of hot start at 95 °C, 45 amplification cycles (95 °C for 10 s, 57 °C for 15 s, 72 °C for 20 s, 78 °C for 1 s with a single fluorescence measurement), a melting curve (60–95 °C at 2.2 °C s^{-1}, and a continuous fluorescence measurement), and a final cooling step. Single peaks in the melting curve analysis confirmed specificity of amplification. Standard curves based on genomic DNA were generated to determine the efficiency of HKG target amplification by real-time PCR. All reactions were performed in two separate sets of experiments using triplicates for each sample, and results are presented as mean values. Expression levels of HKGs were compared using crossing points (CP) based on the 'second derivative maximum' computed by the LIGHTCYCLER 480 software.

Determination of HKG expression stability using BESTKEEPER and GENORM

BESTKEEPER (Pfaffl *et al.*, 2004) and GENORM (Vandesompele *et al.*, 2002) programs were used to compare expression stability of the candidate reference genes under the tested stress conditions. BESTKEEPER uses raw CP values to compare expression stability of HKGs based on a multitude of pair-wise correlation analyses and determines the BESTKEEPER index. The software compares each gene to this index, thus calculating the Pearson correlation coefficient (r) and the correlation probability (P) between the index and the contributing candidate HKG. It also calculates standard deviations (SD) of the CP values, as well as a coefficient of variance (CV) that is expressed as a percentage

on the CP level. The genes with the highest coefficient of correlation and SD ≤ 1.0 are considered to be most stably expressed (Pfaffl *et al.*, 2004). The GENORM module integrated into qbase^{plus} (Biogazesse, Zulte, Belgium) allows calculation of the gene expression stability measure *M*, based on the average pair-wise variation value (*V*) of a single candidate reference gene with all other control genes. Thus, the gene with the lowest *M* value is most stably expressed. The least stably expressed genes are stepwise excluded, and *M* is recalculated. The recommended number of reference genes is determined using *V* with a cutoff of 0.15 as threshold (Vandesompele *et al.*, 2002).

Results

RNA quality assessment using Bioanalyzer revealed high RNA integrity values (RIN scores of 7.3–9.8) for all tested samples. Real-time PCR assays were optimized with regard to primer concentrations (Table 2) and run conditions for each target to assure high amplification specificity and efficiency. Single peaks in melting curve analyses, as well as single product bands on agarose gels, confirmed target-specific amplifications for all primer pairs used. PCR efficiencies ranged from 91% to 98% (Table 2).

Interstrain HKG expression stability was initially assessed among *S. aureus* strains exposed to individual experimental conditions associated with the LB- and CYGP-based osmotic and organic acid stress adaptation models. This revealed that there was high interstrain variation in HKG expression stability under osmotic and acidic stress conditions in comparison with growth under control conditions in both LB and CYGP w/o glucose (Figs 1 and 2; Table 3). Next, we assessed changes in expression levels due to exposure to osmotic or acidic stress (Table 4). In the NaCl stress adaptation model, we found the most stably expressed HKGs were *rplD*, *rpoB*, and *rho* in nutrient-rich (LB) medium, and *rho*, *gyrB*, and *rpoB* in nutrient-deficient (CYGP w/o glucose) medium. Greater variability in HKG expression levels was detected under the combined experimental conditions of the lactic acid stress adaptation model. In the LB-based lactic acid stress adaptation model, *rplD*, *rpoB, gyrB*, and *rho* were ranked as the best four reference genes using BESTKEEPER and GENORM. When we assessed expression stability in the CYGP w/o glucose-based lactic acid stress adaptation model, *rho* represented the most stably expressed candidate reference gene.

Finally, the generated data also allowed us to determine which of the candidate reference genes exhibits the most stable expression across all three tested conditions (Table S3). When including all three conditions, *rplD* was most stably expressed in LB, whereas *rpoB* (BESTKEEPER) and *rho* (GENORM) exhibited the most stable expression in CYGP w/o glucose. In general, we observed less variation in the HKG expression across all conditions in LB than in CYGP w/o glucose.

Discussion

In this study, we investigated the suitability of nine HKGs as reference genes for normalization of qPCR mRNA expression levels in food-related stress adaptation models in *S. aureus*. Our objective was to identify reference genes that could be used as endogenous controls in the development of experimental models for investigation of enterotoxin gene expression changes associated with the exposure and adaptation of enterotoxigenic *S. aureus* strains to NaCl and lactic acid stress encountered during food production and preservation.

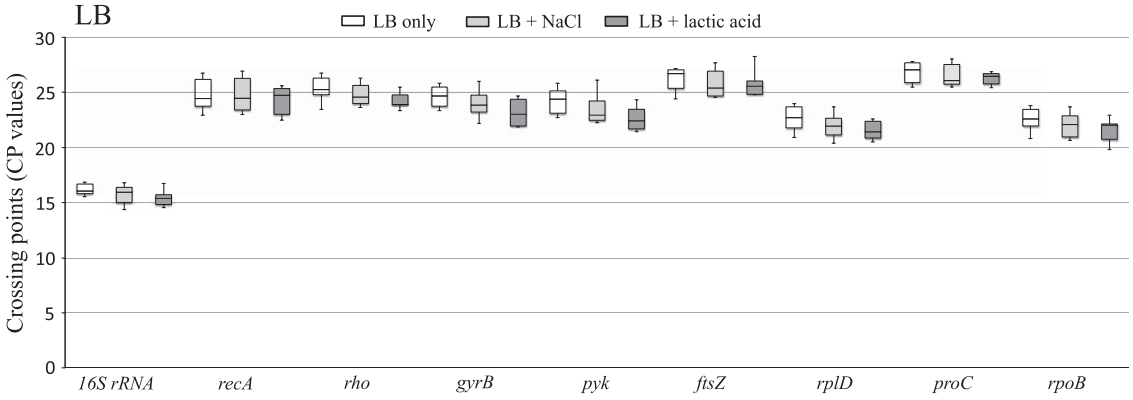

Fig. 1. Changes in CP values of different candidate reference genes when *Staphylococcus aureus* strains were exposed to LB only, LB with 4.5% NaCl, and LB adjusted to pH 6.0 using lactic acid. The graph depicts ranges of HKG expression across the tested strains as well as medians and 25th/75th percentiles.

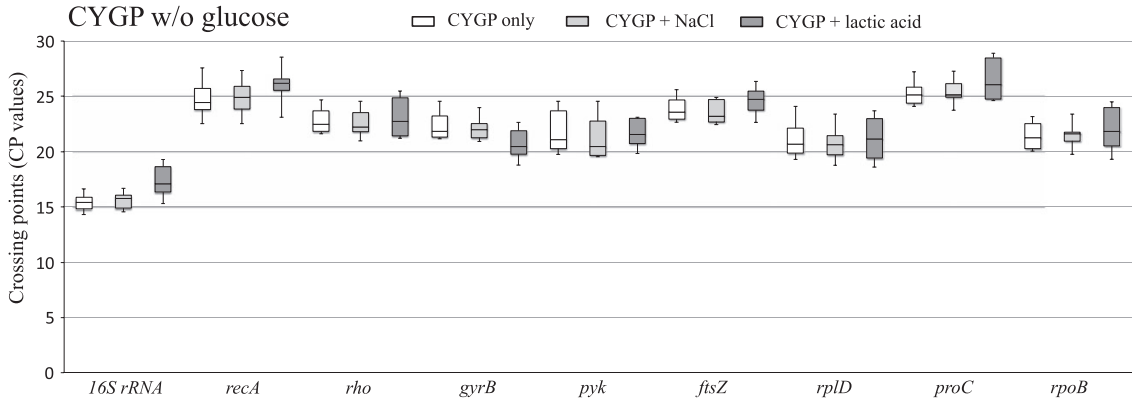

Fig. 2. Changes in CP values of different candidate reference genes when *Staphylococcus aureus* strains were exposed to CYGP only, CYGP with 4.5% NaCl, and CYGP adjusted to pH 6.0 using lactic acid. The graph depicts ranges of HKG expression across the tested strains as well as medians and 25th/75th percentiles.

Table 3. Ranking of reference genes suitable for normalization of experiments comparing expression across strains within a single experimental condition

		Ranking								
Condition	Software	1	2	3	4	5	6	7	8	9
LB	BESTKEEPER	*rplD*	*rpoB*	*gyrB*	*rho*	*pyk*	*ftsZ*	*proC*	*16S rRNA*	
	GENORM	*gyrB*	*rpoB*	*rplD*	*rho*	*pyk*	*proC*	*ftsZ*	*16S rRNA*	*recA*
CYGP	BESTKEEPER	*rplD*	*gyrB*	*rho*	*rpoB*	*recA*	*ftsZ*	*proC*	*16S rRNA*	
	GENORM	*rplD*	*16S rRNA*	*rho*	*proC*	*ftsZ*	*rpoB*			
LB-NaCl	BESTKEEPER	*rplD*	*rho*	*pyk*	*rpoB*	*ftsZ*	*proC*	*gyrB*	*16S rRNA*	
	GENORM	*rho*	*rplD*	*rpoB*	*proC*	*ftsZ*	*pyk*	*gyrB*	*recA*	
CYGP-NaCl	BESTKEEPER	*rho*	*rplD*	*gyrB*	*rpoB*	*proC*	*recA*	*ftsZ*	*16S rRNA*	
	GENORM	*rho*	*rpoB*	*gyrB*	*proC*	*ftsZ*	*rplD*	*recA*		
LB acid	BESTKEEPER	*rplD*	*rpoB*	*pyk*	*recA*	*gyrB*	*rho*	*ftsZ*	*16S rRNA*	*proC*
	GENORM	*rplD*	*pyk*	*rho*	*rpoB*	*gyrB*	*recA*	*ftsZ*	*proC*	*16S rRNA*
CYGP acid	BESTKEEPER	*16S rRNA*	*pyk*	*recA*	*ftsZ*					
	GENORM	*rho*	*rpoB*	*proC*	*ftsZ*					

Only HKGs that met the criteria of SD ≤ 1.0 (BESTKEEPER) and $M \leq 1.0$ (GENORM) were included. For more detailed results, see Table S1.

Table 4. Ranking of reference genes suitable for normalization of stress adaptation models comparing changes in expression levels due to exposure to osmotic stress (4.5% NaCl) or acidic stress (lactic acid, pH 6.0)

		Ranking								
Medium	Software	1	2	3	4	5	6	7	8	9
LB vs. LB + NaCl	BESTKEEPER	*rplD*	*rpoB*	*rho*	*gyrB*	*ftsZ*	*proC*	*16S rRNA*		
	GENORM	*rplD*	*rho*	*rpoB*	*pyk*	*gyrB*	*proC*	*ftsZ*	*recA*	*16S rRNA*
CYGP vs. CYGP + NaCl	BESTKEEPER	*rho*	*gyrB*	*rpoB*	*proC*	*ftsZ*	*16S rRNA*			
	GENORM	*rho*	*rpoB*	*gyrB*	*proC*	*ftsZ*	*rplD*	*recA*		
LB vs. LB + acid	BESTKEEPER	*rplD*	*rpoB*	*gyrB*	*rho*	*proC*	*ftsZ*	*16S rRNA*		
	GENORM	*rplD*	*rho*	*rpoB*	*gyrB*	*pyk*	*proC*	*ftsZ*	*16S rRNA*	*recA*
CYGP vs. CYGP + acid	BESTKEEPER*	*rho*	*proC*	*ftsZ*	*gyrB*					
	GENORM	*rho*	*rpoB*	*proC*	*ftsZ*					

Only HKGs that met the criteria of SD ≤ 1.0 (BESTKEEPER) and $M \leq 1.0$ (GENORM) were included. For more detailed results, see Table S2.
*When all HKGs were included, BESTKEEPER determined that only *ftsZ* would be suitable as a reference gene (SD = 0.96, $r = 0.880$, $P = 0.001$). Results listed depict the ranking of reference genes, when strain KLT_6 leading to the most variable results was excluded.

With this objective in mind, stress adaptation experiments were performed using both a nutrient-rich (LB) and a nutrient-deficient (CYGP w/o glucose) medium adjusted to 4.5% NaCl and pH 6.0 (lactic acid stress) to reflect stress conditions relevant to the food production. Both media do not contain glucose, which was reported to have a negative effect on enterotoxin expression (Regassa *et al.*, 1991). CYGP w/o glucose was selected as a nutrient-deficient medium, after assessing staphylococcal growth in various minimal media, including chemically defined medium (Hussain *et al.*, 1992), *S. aureus* synthetic medium (Gertz *et al.*, 1999), AAM (Rudin *et al.*, 1974), as well as broth containing 10 g L^{-1} hydrolyzed casamino acids and 122 μM tryptophan. However, CYGP w/o glucose represented the only nutrient-deficient medium enabling sufficient growth of all tested strains.

In this study, we evaluated expression stability using both BESTKEEPER and GENORM, applications designed to identify the best-suited reference genes out of a set of HKGs. Rank assignment of the candidate reference genes under the tested experimental conditions varied using these two methods. This is expected because the two applications rely on different assumptions and algorithms for reference gene ranking. Firstly, BESTKEEPER determines overall stability of candidate gene expression by comparison of CP variation based on mean values. Several HKGs exhibited low interstrain variability of expression levels across all growth conditions and tested media (SD ≤ 1 CP), including *16S rRNA* gene, *proC*, *rho*, *rpoB, ftsZ,* and *gyrB. 16S rRNA* gene exhibited the lowest interstrain variation in our study, supported by low SD ($0.41 \leq SD \leq 1.0$) and CV (2.51% $CP \leq CV \leq 6.14\%$) values. While this gene is commonly used in *S. aureus* for normalization of qPCR data due to its high target copy number (Eleaume & Jabbouri, 2004; Lee *et al.*, 2007; Stutz *et al.*, 2011), others have suggested that this gene may not be suitable as a reference gene in *S. aureus* because *16S rRNA* gene transcripts were shown to by far exceed most other transcripts in stability (McKillip *et al.*, 1998) and do not reflect overall mRNA in this organism (Theis *et al.*, 2007). Secondly, BESTKEEPER performs numerous pair-wise correlation analyses between the candidate reference genes and computes highly correlated genes into an index. Subsequently, the software compares each gene to this index, thus calculating the Pearson correlation coefficient (r) and the correlation probability (P) between the index and the contributing candidate HKG.

In contrast, the GENORM algorithm relies on the assumption that the expression ratio of the two most adequate reference genes should be highly similar among all samples irrespective of the tested conditions. The GENORM software also calculates an optimal number of reference genes. In our study, GENORM determined the use of two reference genes (*rplD* and *rho*) to be optimal for normalization in osmotic and acid stress adaptation models that determine expression response changes by comparing gene expression between strains in LB supplemented with NaCl (4.5%) or LB adjusted to pH 6.0 using lactic acid, relative to controls that are exposed to regular LB media. However, this number would not suffice to normalize expression data when similar stress adaptation models are investigated in the nutrient-deficient medium CYGP w/o glucose. For these conditions, the software suggested the use of three reference genes for the NaCl assay and five reference genes for the lactic acid assay.

We evaluated the suitability of nine HKGs as internal controls for normalization of qPCR mRNA expression levels in food-related stress adaptation models in *S. aureus*. Among the set of HKGs tested, *rplD*, *rpoB*, *gyrB*, and *rho* were determined to be most stably expressed in LB and were therefore considered to represent the most suitable reference genes for assays investigating *S. aureus* stress response to osmotic or acidic conditions in this medium. In CYGP w/o glucose, *rho* and *proC* were stably expressed across all tested conditions. We recommend the use of HKGs for normalization of qPCR mRNA expression levels that ranked in high positions in both BESTKEEPER and GENORM rankings under the specific experimental conditions employed. This is the first study presenting comprehensive data on changes in expression of various *S. aureus* HKGs under conditions of osmotic and lactic acid stress, enabling selection of reference genes for qPCR-based stress response models in *S. aureus*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Ranking of reference genes suitable for normalization of experiments comparing expression across strains within one experimental condition only.

Table S2. Ranking of reference genes suitable for normalization of stress adaptation models by comparison of expression levels between one stress condition (either NaCl or acidic stress) and the control condition without stress.

Table S3. Ranking of HKG that exhibited the highest stability over all three tested conditions (NaCl stress, lactic acid stress, control).

5.2 Publication 10

Temporal expression of the staphylococcal enterotoxin D gene under NaCl stress conditions

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RESEARCH LETTER – Food Microbiology

Temporal expression of the staphylococcal enterotoxin D gene under NaCl stress conditions

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One-sentence summary: This piece of research shows a strain-specific variation in the expression of the enterotoxin D gene in *Staphylococcus aureus* under NaCl stress conditions and its control by the regulatory elements *agr*, *sarA* and *sigB*.

Editor: Wolfgang Kneifel

ABSTRACT

Staphylococcus aureus is one of the most osmotolerant food-borne pathogens. While its growth is repressed by competing bacteria, the organism exhibits a growth advantage at increased salt concentrations. Staphylococcal enterotoxin D leads to vomiting and diarrhea upon ingestion. To date, the effect of NaCl on both *sed* expression and its regulatory control are unclear. We determined the impact of NaCl stress on *sed* expression and the influence of *agr*, *sarA* and *sigB* on *sed* expression under NaCl stress. The temporal expression of *sed* in LB and LB with 4.5% NaCl was compared, as well as *sed* expression of wild-type (wt) strains and isogenic Δ*agr*, Δ*sarA* and Δ*sigB* mutants. In general, NaCl stress led to decreased *sed* expression. However, one strain exhibited a trend towards increased *sed* expression under NaCl stress. No significant effect of *agr* on *sed* expression was detected and only one Δ*sigB* mutant showed a significant decrease in *sed* expression in the early stationary phase under NaCl stress. One Δ*sarA* mutant showed decreased *sed* expression in the early stationary and another increased *sed* expression in the stationary growth phase under NaCl stress. These findings suggest high strain-specific variation in *sed* expression and its regulation under NaCl stress.

Key words: *Staphylococcus aureus*; *sed*; NaCl stress; regulation; stress response

INTRODUCTION

Staphylococcus aureus is the most prevalent pathogen implicated in food-borne intoxications worldwide. Strains can produce one or several of the five major staphylococcal enterotoxins (SE), designated SEA-SEE. Upon consumption of SE preformed in food, patients suffer from violent vomiting, diarrhea and abdominal cramping. In the US alone, an estimated 240 000 cases of staphylococcal food poisoning occur each year (CDC), leading to hopitalization in 1000 of cases and to six deaths (Scallan *et al.* 2011). While its growth is repressed by competing bacteria in most food matrices, *S. aureus* has a crucial growth advantage under NaCl stress (Chapman 1945; Troller 1986). The organism is able to grow at a_w values < 0.90 (2.6 M NaCl), rendering it one of the most osmotolerant food-borne pathogens (Troller 1986). While the osmoadaptive process in *S. aureus* is comparable to that in other pathogens, regulatory mechanisms controlling NaCl stress response do not seem to follow common patterns. For instance, NaCl stress induces the expression of the alternative sigma factor σ^B in *Bacillus subtilis*, whereas 1 M NaCl was shown to repress σ^B in *S. aureus* (Chan *et al.* 1998).

Several studies evaluated SE expression under NaCl stress using immunological methods (Genigeorgis and Sadler 1966; McLean, Lilly and Alford 1968; Genigeorgis *et al.* 1971; Troller 1971; Ewald and Notermans 1988). For a comprehensive review on enterotoxin production under stress conditions, see Schelin *et al.* (2011). However, the value of these data is limited, because it was later reported that loss of serological recognition does not equal loss of emetic activity (Bennett 2005). It has therefore

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been suggested that the evaluation of enterotoxin expression on mRNA level would represent a more useful tool that allows to determine the risk of *S. aureus* food poisoning (Lee *et al.* 2007).

Staphylococcal enterotoxin D (SED) is frequently detected in *S. aureus* strains associated with intoxications (Wieneke, Roberts and Gilbert 1993; Kérouanton *et al.* 2007). Transcription of *sed* is moderately increased in the post-exponential growth phase when no stress is applied (Tseng, Zhang and Stewart 2004; Derzelle *et al.* 2009). Investigating the effect of various regulatory factors on *sed* expression, Tseng, Zhang and Stewart (2004) were able to show that *sed* promoter activity is increased by the accessory gene regulator (Agr) and the staphylococcal accessory regulator (SarA) and decreased by σ^B and the repressor of toxins. The function of the regulatory factors has also been shown to be highly connected (Yarwood and Schlievert 2003; Hsieh, Tseng and Stewart 2008). The post-exponential increase in *sed* transcription was shown to result from an Agr-dependent decrease in Rot activity rather than from a direct effect of Agr on *sed* expression (Tseng, Zhang and Stewart 2004). It has been suggested that SED is only partially upregulated by RNAIII and that high concentrations of the enterotoxin can be produced independently of *agr* (Yarwood and Schlievert 2003).

To date, both the effect of NaCl stress on *sed* expression and the regulatory mechanisms controlling *sed* expression under NaCl stress remain unclear. Therefore, we aimed to (i) determine *sed* mRNA levels during growth of *S. aureus* under NaCl stress conditions encountered during food production and preservation and (ii) evaluate the influence of *agr, sarA* and *sigB* on *sed* expression under NaCl stress.

MATERIALS AND METHODS

Bacterial strains

All *S. aureus* strains used in this study are listed in Table 1. Five *sed*$^+$ isolates (BW10, KLT8, RKI1, RKI2, SAI48) were selected. Three of these strains were used to create regulatory mutants and to assess the influence of *agr, sarA* and *sigB* on *sed* expression under NaCl stress. Strains RN27, NM518, BB1385 and LR12 were kindly provided by Brigitte Berger-Bächi (University of Zurich). Regulatory knockout mutants were obtained by transduction of the *agr, sarA* and *sigB* knockouts from NM518, BB1385 and LR12 to the isolates RKI1, RKI2 and SAI48, using phage 80α and protocols previously described (Charpentier *et al.* 2004). Antibiotics were used at the following concentrations to screen for presumptive regulatory knockout mutants using selective plates: tetracycline at 10 μg mL^{-1} and erythromycin at 10 μg mL^{-1} (Sigma). Correct deletion of the respective regulatory genes in putative mutants grown on selective plates was evaluated by PCR screening for *agr, sarA* and *sigB*. In addition, characteristic changes in phenotype associated with loss of the regulatory element were observed such as changes in hemolysis pattern or loss of pigmentation.

Bacterial growth and collection of samples

S. aureus strains were grown in LB broth (Bertani 1959) and LB broth adjusted to 4.5% NaCl (0.8 M NaCl, $a_w = 0.97$). These conditions were chosen to mimic NaCl levels encountered during production or preservation of foods. Media ingredients were obtained from Difco laboratories (Detroit, MI), Oxoid (Cambridge, UK), Becton Dickinson (Allschwil, Switzerland) and Sigma (Buchs, Switzerland). Growth phases were determined using plate count and DMFit 3.0 (Baranyi and Roberts 1994).

Frozen stock cultures were resuscitated by plating on 5% sheep blood agar and incubated at 37°C over night. Single colonies were grown in 5 mL of LB broth for 18 h (37°C, 225 rpm shaking) to reach stationary phase. Aliquots of 1 mL of the overnight cultures were centrifuged (6000 × g for 10 min) and washed twice with 0.8% NaCl to remove residual media components. We prepared 10-fold dilution series and used 50 μL of a 10^{-3} dilution to inoculate a 50 mL day culture of LB or LB adjusted to 4.5% NaCl. For RKI2Δ*sarA*, the 10^{-2} dilution was used instead of the 10^{-3} dilution to account for an extended lag phase in this strain. Day cultures were grown at 225 rpm and 37°C to early exponential (T1), mid-exponential (T2), early stationary (T3) and stationary growth phase (T4). Cells were harvested by centrifugation at 8000 × g for 5 min and resuspended in 500 μL RNA protect bacteria reagent (Qiagen, Hombrechtikon, Switzerland), incubated at room temperature for 5 min and harvested again by centrifugation (3000 × g for 5 min). Subsequently, cell pellets were stored at −80°C. This procedure was repeated to gain two independent samples of each strain grown under control conditions (LB), as well as NaCl stress conditions (LB adjusted to 4.5% NaCl).

RNA extraction and reverse transcription

Cell lysis, RNA extraction and reverse transcription were performed as previously described (Sihto *et al.* 2014). RNA integrity numbers determined by Bioanalyzer (Agilent Technologies, Waldbronn, Germany) ranged from 7.2 to 9.9.

Real-time quantitative PCR (qPCR)

Single peaks in the melting curve analyses and single product bands on agarose gels confirmed target-specific amplifications for all primers (Table 2). Real-time PCR experiments were performed using LightCycler480 (Roche). A total reaction volume of 10 μL was used, including 4 μL cDNA template (dilution 1:100), 250 nM of each primer and the LightCycler480 SYBR Green I master mix (Roche). Water (no template) and RT minus samples were used as controls. An inter-run calibrator sample was included as a control for variation in cDNA synthesis and amplification. PCR cycling conditions included 8 min at 95°C, 45 amplification cycles (95°C for 10 s, the respective annealing temperature for 15 s, 72°C for 20 s, 78°C for 1 s with a single fluorescence measurement), a melting curve (60–95°C at 2.2°C s^{-1}, and a continuous fluorescence measurement) and a final cooling step. Standard curves based on genomic DNA were generated to determine the efficiency of target gene amplification for each strain. All samples were amplified in triplicates. Expression levels of *sed* were compared using relative expression ratios, which were normalized using *rho* and *rplD* as reference genes, previously shown to be suitable for normalization of *S. aureus* qPCR data in LB and LB adjusted to 4.5% NaCl (Sihto *et al.* 2014). The effect of NaCl on *sed* expression was assessed by calculating fold changes in *sed* expression in early exponential, mid-exponential, early stationary and stationary phase relative to the mean *sed* expression level at T1 in LB. The effect of *agr, sarA* and *sigB* on *sed* expression was assessed by calculating fold changes in *sed* expression in isogenic regulatory mutants relative to mean *sed* expression in the wt at T1 in LB.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (SPSS Inc., Chicago, IL). Results were considered significant at

Table 1. *S. aureus* wt and mutant strains used in this study.

Strain ID	Relevant characteristics	Source (reference)
RKI1	Clinical strain associated with food-borne outbreak, CC8/t648, *sea+, sed+, sej+*	Robert Koch Institute, Germany
RKI2	Clinical strain associated with food-borne outbreak, CC8/t008, *sea+, sed+, sej+*	Robert Koch Institute, Germany
SAI48	Clinical strain isolated from *S. aureus* infection, CC5/t002, *sec+, sed+, sej+*	Institute of Medical Microbiology, University of Zurich, Switzerland
KLT8	Clinical strain associated with foodborne outbreak, CC5/t8017 *sea+, sed+, sej+, egc cluster*	Cantonal Laboratory Thurgau, Switzerland
BW10	Clinical strain associated with foodborne outbreak, CC45/t383, *sec+, sed+, sej+*	Medical Department of the German Federal Armed Forces, Germany
NM518	RN4220_*agr*::*ermB*	Brigitte Berger-Bächi (McCallum, Hinds and Ender 2010)
LR12	RN4220_*sarA*::*tetL*	Brigitte Berger-Bächi (McCallum, Hinds and Ender 2010)
BB1385	BB255_rsbUVWσ^B::*ermB*	Brigitte Berger-Bächi (Kullik, Giachino and Fuchs 1998)
RN27	80α lysogen	Brigitte Berger-Bächi (Novick 1963)
RN4220	Restriction deficient mutant of strain NCTC8325-4 that accepts foreign DNA	Brigitte Berger-Bächi (Kreiswirth *et al.*1983)
RKI1Δ*agr*	RKI1 with *ermB* replacing *agr*	This work
RKI1Δ*sigB*	RKI1 with *ermB* replacing *sigB*	This work
RKI1Δ*sarA*	RKI1 with *tetL* replacing *sarA*	This work
RKI2Δ*agr*	RKI2 with *ermB* replacing *agr*	This work
RKI2Δ*sigB*	RKI2 with *ermB* replacing *sigB*	This work
RKI2Δ*sarA*	RKI2 with *tetL* replacing *sarA*	This work
SAI48Δ*agr*	SAI48 with *ermB* replacing *agr*	This work
SAI48Δ*sigB*	SAI48 with *ermB* replacing *sigB*	This work
SAI48Δ*sarA*	SAI48 with *tetL* replacing *sarA*	This work

Table 2. Primer pairs, including amplicon sizes, E-values and annealing temperatures for the target and reference genes used in this study.

Gene	Function	Primer pair (5′–3′)	Amplicon size (bp)	E(%)	Annealing T (°C)	Reference
sed	Staphylococcal enterotoxin D	GTG GTG AAA TAG ATA GGA CTG C ATA TGA AGG TGC TCT GTG G	384	90–8	62	(Monday and Bohach 1999)
rho	Transcription termination factor	GAA GCT GCT GAA GTC G CGT CCA TAC GTG AAC CC	319	97–8	57	(Sihto *et al.* 2014)
rplD	Ribosomal protein L4	TTC GGA CCA ACT CCA AGA CGA GCA CCT CCT CAA C	352	93–6	57	(Sihto *et al.* 2014)

$P < 0.05$. Growth parameters such as lag phase duration, exponential phase growth rate and maximum cell density in stationary phase were compared using one-way ANOVA. Log transformed relative expression ratios of *sed* were also compared using one-way ANOVA.

RESULTS

Effect of NaCl stress on growth

Bacterial growth of the five wt strains was compared in LB and LB adjusted to 4.5% NaCl (Fig. 1). Respective isogenic *agr, sarA* and *sigB* mutant strains of three wt strains grew similarly with exception of RKI2Δ*sarA* growing more slowly under NaCl stress (Fig. S1, Supporting Information). OD values at sampling time points were as follows: 0.01 (T1), 0.15 (T2), 1.80 (T3) and >2.00 (T4). In the wt strains grown under NaCl stress, the mean lag phase duration was prolonged (Δ1.02 ± 0.73 h; $P = 0.01$), the growth rate was decreased (Δ0.15 ± 0.10 lg CfU mL^{-1} h^{-1}; $P = 0.02$) and cultures reached lower maximum cell densities (Δ0.72 ± 0.42 lg CfU mL^{-1}; $P = 0.00$). Detailed data on the effect of NaCl stress on lag phase duration, growth rates and maximum cell densities for *agr, sarA* and *sigB* mutant strains is provided as supplemental material (Table S1, Supporting Information).

Effect of NaCl stress on *sed* expression

The impact of 4.5% NaCl on temporal *sed* expression is presented in Fig. 2 and fold change values are provided in Table 3. In both LB and LB adjusted to 4.5% NaCl, highest *sed* expression was generally observed in the stationary growth phase. However, in strain

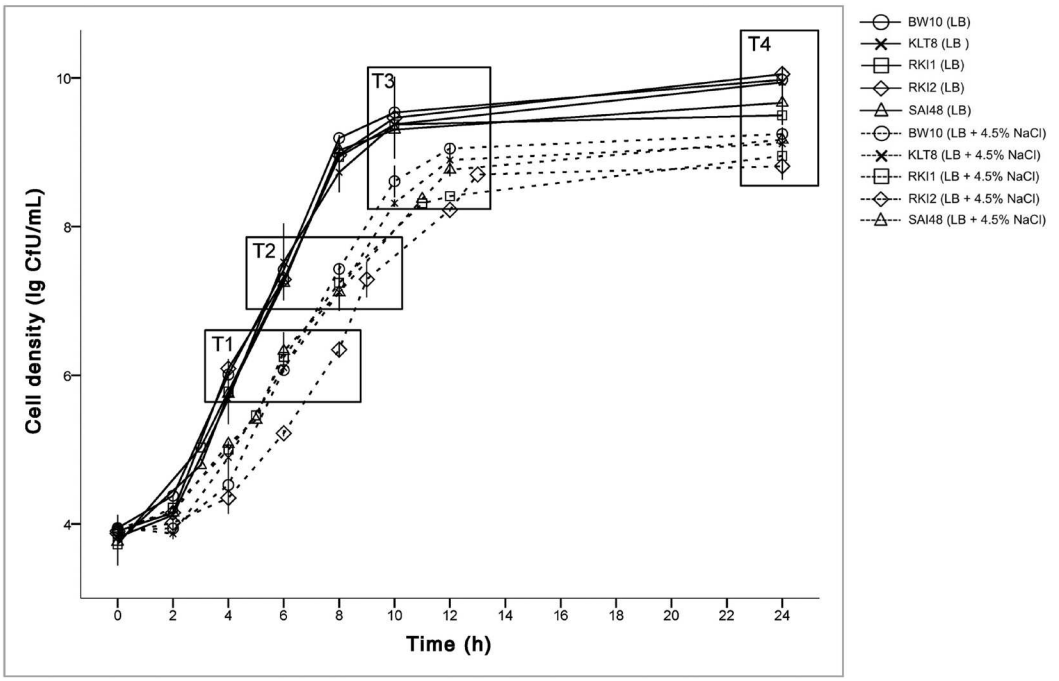

Figure 1. Growth of five *S. aureus* strains (BW10, KLT8, RKI1, RKI2, SAI48) was compared in LB and LB adjusted to 4.5% NaCl. Cells were harvested in early exponential (T1), mid-exponential (T2), early stationary (T3) and stationary growth phase (T4).

Table 3. Effect of NaCl stress on *sed* expression. Fold change of *sed* expression in early exponential (T1), mid-exponential (T2), early stationary (T3) and stationary phase (T4) is indicated relative to the mean expression level at T1 in LB. Statistically significant changes in *sed* expression in LB + 4.5% NaCl compared to LB only are marked by an asterisk.

Strain	LB				LB + 4.5% NaCl			
	T1	T2	T3	T4	T1	T2	T3	T4
BW10	1.0 (± 0.7)	3.3 (± 1.7)	26.4 (± 3.0)	337.3 (± 3.0)	1.6 (± 0.2)	2.2 (± 0.9)	21.2 (± 6.1)	16.1 (± 4.2)* ↓
KLT8	1.0 (± 0.6)	1.0 (± 0.3)	12.7 (± 6.4)	56.4 (± 13.3)	0.5 (± 0.2)	0.5 (± 0.0)	3.2 (± 1.0)* ↓	9.3 (± 2.3)* ↓
RKI1	1.0 (± 0.4)	1.9 (± 2.1)	7.0 (± 5.4)	98.9 (± 70.0)	0.6 (± 0.2)	0.7 (± 0.6)	6.0 (± 1.4)	13.3 (± 6.0)* ↓
RKI2	1.0 (± 0.8)	1.7 (± 2.0)	6.9 (± 4.9)	209.3 (± 124.7)	0.9 (± 0.7)	0.9 (± 0.6)	4.7 (± 2.4)	19.5 (± 13.3)* ↓
SAI48	1.0 (± 0.8)	4.5 (± 4.9)	17.5 (± 10.6)	97.9 (± 53.7)	1.1 (± 0.3)	3.3 (± 2.4)	33.1 (± 20.4)	128.7 (± 54.9)

BW10, *sed* expression under NaCl stress peaked in early stationary phase.

In all growth phases, relative *sed* expression values tended to be lower under NaCl stress compared with no-stress conditions. Still, only in the stationary growth phase, a statistically significant reduction in *sed* expression under NaCl stress was observed for four out of five tested strains ($P = 0.00$). Interestingly, one strain (SAI48) exhibited a trend towards increased *sed* expression under NaCl stress in the stationary growth phase.

Effect of *agr*, *sarA*, *sigB* on *sed* expression in LB

Temporal relative expression ratios of *sed* were determined for wt strains and their isogenic Δ*agr*, Δ*sarA* and Δ*sigB* knockout mutant strains. Fold change in *sed* expression was calculated relative to the mean *sed* expression level at early exponential phase in the respective wt (Table 4). No significant changes in *sed* expression were found when comparing *sed* expression in wt strains and isogenic Δ*agr* mutants. This was the case for both growth in LB and growth in LB adjusted to 4.5% NaCl. As for the effect of NaCl on *sed* expression in the Δ*agr* mutants, NaCl stress only led to a significant reduction in *sed* expression in the stationary growth phase for one of the three tested strains (RKI2Δ*agr*, $P = 0.00$). When assessing the influence of *sarA* on *sed* expression, the effect of Δ*sarA* mutations was dependent of the growth phase and the strain. In SAI48 at early stationary phase under NaCl stress, a statistically significant decrease in *sed* expression was present in the Δ*sarA* mutant compared to the wt ($P = 0.02$). By contrast, in RKI1 at stationary phase under NaCl stress, a statistically significant increase in *sed* expression in the Δ*sarA* mutant compared to the wt ($P = 0.02$) was observed. As regards the effect of NaCl on *sed* expression in the Δ*sarA* mutants, NaCl stress only led to a significant reduction in *sed* expression in the early stationary growth phase for one of the three tested strains (RKI1Δ*sarA*, $P = 0.01$). When assessing the effect of Δ*sigB*, a statistically significant decrease in *sed* expression in the early stationary phase under NaCl stress in the Δ*sigB* mutant of SAI48 compared to the SAI48 wt ($P = 0.02$) was observed.

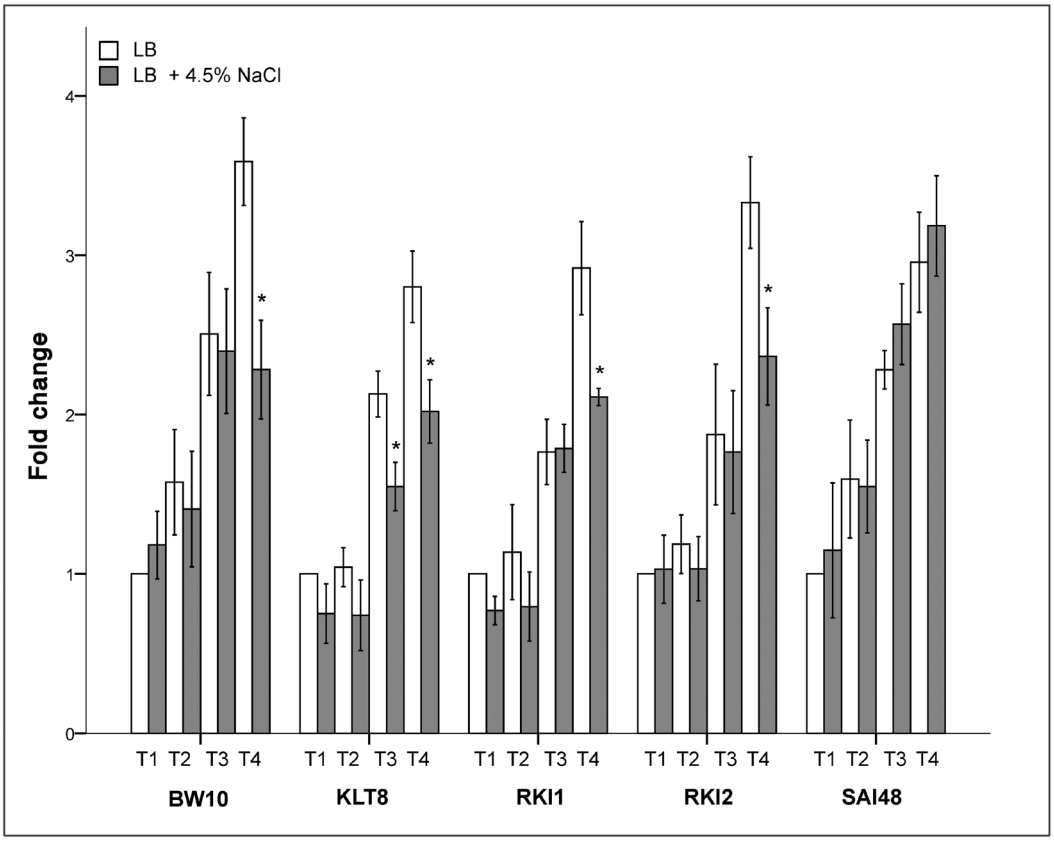

Figure 2. Effect of NaCl stress on *sed* expression in five *S. aureus* strains (BW10, KLT8, RKI1, RKI2, SAI48). Fold change of *sed* expression in early exponential (T1), mid-exponential (T2), early stationary (T3) and stationary phase (T4) was calculated relative to the mean expression level at T1 in LB. Statistically significant changes in *sed* expression in LB adjusted to 4.5% NaCl compared to LB ($P < 0.05$) are marked by an asterisk.

Table 4. Effect of *agr*, *sarA* and *sigB* on *sed* expression. Fold change of *sed* expression in wt strains and isogenic regulatory mutants in early exponential (T1), mid-exponential (T2), early stationary (T3) and stationary phase (T4) is presented relative to mean *sed* expression in the wt at T1 in LB. Statistically significant changes in *sed* expression between wt and mutants are marked by an asterisk.

Mutation	Strain	LB				LB + 4.5% NaCl			
		T1	T2	T3	T4	T1	T2	T3	T4
Δ*agr*	RKI1	1.0 (± 0.8)	2.0 (± 1.2)	14.7 (± 11.9)	26.7 (± 18.6)	0.9 (± 0.7)	1.4 (± 0.8)	5.5 (± 3.9)	21.0 (± 16.8)
	RKI2	1.0 (± 0.2)	0.9 (± 0.3)	14.1 (± 13.5)	335.5 (± 181.1)	0.4 (± 0.1)	0.5 (± 0.2)	3.4 (± 2.6)	6.2 (± 2.8)
	SAI48	3.1 (± 2.0)	5.9 (± 3.2)	47.7 (± 24.7)	130.1 (± 20.1)	4.2 (± 2.3)	4.4 (± 1.6)	28.4 (± 13.9)	49.6 (± 39.0)
Δ*sarA*	RKI1	0.5 (± 0.3)	0.6 (± 0.0)	7.9 (± 1.0)	78.3 (± 5.7)	0.3 (± 0.0)	0.5 (± 0.2)	1.8 (± 0.6)	53.3 (± 12.2)* ↑
	RKI2	0.4 (± 0.2)	0.7 (± 0.0)	15.4 (± 11.0)	103.2 (± 43.6)	0.3 (± 0.3)	0.6 (± 0.4)	2.9 (± 0.6)	48.9 (± 16.3)
	SAI48	0.7 (± 0.3)	1.6 (± 0.6)	12.1 (± 2.0)	186.4 (± 53.5)	2.0 (± 1.4)	1.6 (± 0.6)	5.9 (± 1.5)* ↓	161.9 (± 68.2)
Δ*sigB*	RKI1	0.5 (± 0.3)	1.3 (± 0.8)	5.1 (±1.2)	129.6 (± 51.1)	0.5 (± 0.2)	0.6 (±0.2)	1.7 (± 0.8)	28.1 (± 17.0)
	RKI2	2.2 (± 3.7)	1.1 (± 1.3)	4.0 (± 2.6)	46.0 (± 17.8)	0.7 (± 0.8)	1.1 (± 1.5)	3.7 (± 3.8)	21.5 (± 7.2)
	SAI48	2.5 (± 1.0)	2.2 (± 0.7)	17.4 (± 6.4)	292.9 (± 153.8)	1.5 (± 0.8)	1.3 (± 0.2)	5.8 (± 2.1)* ↓	41.5 (± 13.7)

Concerning the effect of NaCl on *sed* expression in the Δ*sigB* mutants, NaCl stress led to a significant reduction in *sed* expression in the stationary growth phase for two of the three tested strains (RKI1Δ*sigB* and SAI48Δ*sigB*, $P \leq 0.02$).

DISCUSSION

This is the first study evaluating the effect of NaCl stress on *sed* expression in *S. aureus*. The selected NaCl level mimics stress conditions encountered during production and preservation of many food items, including various raw ham or cheese products. These stress conditions grant *S. aureus* a competitive growth advantage over other microorganisms present in the food matrix. Under 4.5% NaCl stress, we detected prolonged lag phases, as well as a decrease in growth rates and maximum cell densities. This is consistent with previous studies showing a linear correlation between growth of *S. aureus* and NaCl concentration (Ewald and Notermans 1988; Genigeorgis 1989).

Many genes coding for secreted proteins are upregulated at the end of the exponential growth phase, followed by downregulation of housekeeping and facultative genes during the transition to stationary growth phase and preparation of cells for long-term survival during full stationary growth phase (Derzelle *et al.* 2009). In both LB broth and LB broth adjusted to 4.5% NaCl, highest *sed* expression was generally observed in the stationary phase of growth showing a moderate post-exponential increase. In contrast to the plasmid encoded *sed,* expression of the chromosomally encoded *agr*-regulated enterotoxin genes *seb* and *sec* has been shown to exhibit a more pronounced post-exponential increase (Derzelle *et al.* 2009).

NaCl has been reported to affect production of SEA, SEB and SEC. In this study, NaCl stress resulted in decreased *sed* expression. However, one strain exhibited a trend towards increased *sed* expression under NaCl stress. Considering the pronounced strain-specific variation detected in this study, this suggests that NaCl may induce *sed* expression in a subset of *S. aureus* strains.

In this study, no statistically significant alterations in *sed* expression were observed in Δ*agr,* Δ*sarA* or Δ*sigB* mutants compared to their isogenic wt, when strains were grown without NaCl stress. Previous studies suggested that *sed* expression is increased by Agr and SarA, and decreased by σ^B and Rot (Bayles and Iandolo 1989; Tseng, Zhang and Stewart 2004). However, these studies used derivatives of NCTC8325, a strain that was shown to exhibit an 11-base deletion in *rsbU*, a gene encoding an indirect positive regulator of σ^B (Gertz *et al.* 1999). As σ^B influences other global regulators including *sar* and *agr,* these findings may be misleading (Bischoff and Entenza 2001). In addition, pronounced strain-specific variation in *S. aureus* is well described, with a study reporting strain-specific variation in Rot levels (Jelsbak *et al.* 2010). As the *agr* effect on *sed* expression was suggested to be Rot-dependent (Tseng, Zhang and Stewart 2004), this may contribute to differences in *sed* expression.

Under the NaCl stress conditions tested in this study (0.8 M NaCl), no significant effect of *agr* on *sed* expression was detected. Similar findings were reported for enterotoxin C (Regassa and Betley 1993), for which a decrease in *sec* mRNA levels under NaCl stress conditions (1.2 M NaCl) compared to control conditions (0 M NaCl) was shown, independent of an intact *agr* allele. In Δ*sarA* mutants grown under NaCl stress, strain-specific variation was observed. One Δ*sarA* mutant showed decreased *sed* expression in the early stationary and another Δ*sarA* mutant increased *sed* expression in the stationary growth phase. Blevins *et al.* (2002) reported strain-specific variation in the regulatory role of *agr* and *sarA*. These findings suggest that this may also be the case for the *agr* and *sarA* regulation under NaCl stress conditions. As for the effect of *sigB* on *sed* expression under NaCl stress, a statistically significant decrease in *sed* expression in the early stationary phase was detected in one of the three tested Δ*sigB* mutant strains. High levels of NaCl were shown to release and activate σ^B (Betley, Borst and Regassa 1992; Miyazaki *et al.* 1999), which in turn influences the regulators SarA and RNAIII (Bischoff and Entenza 2001).

CONCLUSIONS

The data generated in this study indicate that *sed* expression under NaCl stress is controlled by a complex intertwined network of regulatory elements. Therefore, deletion of a single regulatory element does not necessarily alter *sed* expression. While NaCl stress generally leads to decreased *sed* expression in *S. aureus*, our results indicate that it may induce *sed* expression in some strains. As our findings are based on experiments in culture media, additional experiments investigating *sed* expression in the food matrix are needed to determine the effect of stressors encountered during food production and preservation on *sed* expression.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSLE online.

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Conflict of interest statement. None declared.

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5.3 Publication 11

Growth behavior and temporal enterotoxin D expression of *Staphylococcus aureus* strains under glucose and lactic acid stress

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Short communication

Growth behavior and temporal enterotoxin D expression of *Staphylococcus aureus* strains under glucose and lactic acid stress

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ABSTRACT

Ingestion of the staphylococcal enterotoxin D (SED) leads to staphylococcal food poisoning, the most prevalent foodborne intoxication worldwide. Patients suffer from acute signs of gastroenteritis such as violent vomiting, diarrhea, cramps, and fever. As the symptoms result in pronounced electrolyte imbalances and dehydration, the intoxication is particularly dangerous to children and the elderly. SED is formed during growth of *Staphylococcus aureus* in food. While growth of *S. aureus* is repressed by competing bacteria in most food matrices, the organism exhibits a crucial competitive growth advantage in foods with low pH or a low a_w value (*e.g.* through high sugar concentrations). To date, the effect of these stress conditions on *sed* expression is unclear. The objective of this study was to determine *sed* mRNA expression levels of *S. aureus* exposed to glucose and lactic acid stress conditions similar to food production and preservation. To this end, temporal *sed* mRNA expression levels of three *S. aureus* strains grown at control conditions, glucose stress conditions (30% glucose), and lactic acid stress conditions (pH 6.0) were determined using quantitative Real-Time PCR. Under both glucose and acid stress conditions, the mean lag phase duration was prolonged and maximum cell density in late stationary phase was decreased. In addition, glucose stress slightly increased the growth rate of the tested strains and led to decreased *sed* expression in late stationary phase. Lactic acid stress had no statistically significant effect on *sed* expression. Our study provides data on the effect of critical food-related stressors on growth and SE expression of *S. aureus,* which can be used for risk assessment.

1. Introduction

The Centers for Disease Control and Prevention estimate that a total of 240 000 cases of staphylococcal food poisoning (SFP) occur each year in the US, leading to hospitalization in 1000 cases and to six deaths (Scallan et al., 2011). SFP is caused by oral intake of enterotoxins such as staphylococcal enterotoxin D (SED), which are preformed by *Staphylococcus aureus* during growth. While growth of *S. aureus* is repressed by competing bacteria in food matrices, the organism exhibits a crucial growth advantage under stress conditions encountered in many foods, including mildly acidic conditions and low a_w values due to high sugar concentrations (Troller, 1986). As food processing techniques are constantly adapted to reduce costs and meet new consumer demands, knowledge on the effect of critical food-related stressors on growth and SE expression is needed to prevent outbreaks and advance quantitative microbial risk assessment (Schelin et al., 2011).

While some data on the effect of stressors on enterotoxin formation is available, most studies relied on immunological methods for enterotoxin detection (Genigeorgis, Foda, Mantis, & Sadler, 1971; Genigeorgis & Sadler, 1966). However, it was later suggested that quantification of enterotoxin expression on mRNA level represents a more useful tool to determine SFP risk (Lee, Moon, Park, Chang, & Kim, 2007), as loss of serological recognition of SEs does not equal loss of emetic activity (Bennett, 2005).

To date, the effects of glucose and lactic acid stress on *S. aureus* growth and *sed* expression are unclear. Therefore, we aimed to determine *sed* mRNA expression levels of *S. aureus* exposed to glucose and lactic acid stress conditions similar to food production and preservation.

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Table 1
S. aureus strains used in this study.

Strain ID	Relevant characteristics	Source
RKI1	Clinical strain associated with foodborne outbreak, CC8/t648, *sea+*, *sed+*, *sej+*	Robert Koch Institute, Germany
RKI2	Clinical strain associated with foodborne outbreak, CC8/t008, *sea+*, *sed+*, *sej+*	Robert Koch Institute, Germany
SAI48	Clinical strain isolated from *S. aureus* infection, CC5/t002, *sec+*, *sed+*, *sej+*	Institute of Medical Microbiology, University of Zurich, Switzerland

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. aureus strains used in this study are listed in Table 1. Strains were grown in LB broth (Difco laboratories, Detroit, MI), in LB that was supplemented with 30% of glucose (1.6 M, $a_w = 0.96$) (Sigma–Aldrich, Buchs, Switzerland), or lactic acid (pH 6.0, 8 mM) (Sigma–Aldrich, Buchs, Switzerland). The pH in LB control (pH 7.0) and LB lactic acid media was stabilized by inclusion of 100 nM 2-morpholinoethanesulfonic acid hydrate (MES hydrate) (Sigma–Aldrich, Buchs, Switzerland) and the final pH was adjusted using 10 M NaOH (Sigma–Aldrich, Buchs, Switzerland). Growth was monitored by viable cell count of serial dilutions in 0.85% NaCl (Sigma–Aldrich, Buchs, Switzerland) on plate count agar (Oxoid, Cambridge, UK). Growth parameters were calculated using DMFit 3.0 (Baranyi & Roberts, 1994).

Single colonies were transferred from 5% sheep blood agar to 5 mL of LB broth and grown for 18 h (37 °C, 225 rpm). Aliquots of 1 mL of the overnight cultures were centrifuged (6000 × g for 10 min) and washed twice with 0.8% NaCl to remove residual media components. Day culture was inoculated with 10^{-3} dilution of washed overnight culture to result in approximate cell density of 10^4 CFU/mL and incubated at 37 °C, 225 rpm. Cells were harvested by centrifugation (8000 × g for 5 min) at early exponential (T1), mid-exponential (T2), early stationary (T3), and late stationary growth phase (T4). Cells were resuspended in 500 μL RNA protect bacteria reagent (Qiagen, Hombrechtikon, Switzerland), incubated at room temperature for 5 min and harvested again by centrifugation (3000 × g for 5 min). Cell pellets were stored at −80 °C before RNA extraction. The procedure was repeated to gain two independent samples of each strain, condition, and time point.

2.2. Analysis of sed gene expression

RNA was isolated and converted to cDNA by reverse transcription as previously described (Sihto, Tasara, Stephan, & Johler, 2014). RNA samples were quantified and quality controlled using the Nanodrop and Bioanalyzer instruments, respectively. The RNA integrity numbers determined for the samples using the Bioanalyzer (Agilent Technologies, Waldbronn, Germany) ranged from 7.1 to 9.1. For each sample, 100 ng of RNA was converted to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). Reverse transcription was performed twice for each sample. Residual DNA contamination was ruled out in each RNA sample by including a control in which the RT enzyme (RT minus) was omitted. Quantitative PCR was performed using the SYBR Green I kit (Roche Molecular diagnostics, Penzburg, Germany) and the LightCycler 480 (Roche) instrument. Quantification was performed using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics). Primer sequences, primer concentrations, and annealing temperatures are specified in Table 2. BestKeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004) and geNorm (Vandesompele et al., 2002) programs were used to compare expression stability of the candidate reference genes as previously described (Sihto et al., 2014). The influence of glucose and lactic acid stress on *sed* expression in each strain was expressed as both relative expression values and fold changes in *sed* expression in ea rly exponential, mid-exponential, early stationary, and late stationary phase relative to *sed* expression level during early exponential growth (T1) in LB in the respective strain.

2.3. Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (SPSS Inc., Chicago, IL). Results were considered significant at $p < 0.05$. Growth parameters such as lag phase duration, growth rate, and maximum cell density were compared using one-way ANOVA. Log transformed relative expression ratios of *sed* were also compared using one-way ANOVA. Strain-specific differences in *sed* expression were determined using Student's t-test.

3. Results

3.1. Reference gene validation

The expression stability of nine different reference genes was investigated under glucose and lactic acid stress in eight *S. aureus* strains. Based on BestKeeper and geNorm analysis, *gyrB*, *ftsZ*, *pyk* were the most stably expressed reference genes under glucose stress, while *rho*, *rplD*, *rpoB* were most stable under lactic acid stress. Three reference genes were chosen for normalization of

Table 2
Primer pairs, including amplicon sizes, E-values, and annealing temperatures for the target and reference genes used in this study.

Gene	Function	Primer pair (5′ − 3′)	Amplicon size (bp)	Primer c (nM)	E (%)	Annealing T (°C)	Reference
sed	Staphylococcal enterotoxin D	GTG GTG AAA TAG ATA GGA CTG C ATA TGA AGG TGC TCT GTG G	384	250 250	90–8	62	(Monday & Bohach, 1999)
rho	Transcription termination factor	GAA GCT GCT GAA GTC G CGT CCA TAC GTG AAC CC	319	250 250	97–8	57	(Sihto et al., 2014)
rplD	Ribosomal protein L4	TTC GGA CCA ACT CCA AGA CGA GCA CCT CCT CAA C	352	250 250	93–6	57	(Sihto et al., 2014)
gyrB	DNA gyrase (subunit B)	GTC GAA GGG GAC TCT G GCT CCA TCC ACA TCG G	242	250 250	91–6	57	(Sihto et al., 2014)
ftsZ	Cell division protein	TAT TAC TGG TGG CGA GTC A AGT ATT TAC GCT TGT TCG GA	223	250 250	91–3	57	(Sihto et al., 2014)
pyk	Pyruvate kinase	GCT AGT GAC GTT GCC A ATA GTA CGT GCC GTT G	284	500 500	91–7	57	(Sihto et al., 2014)

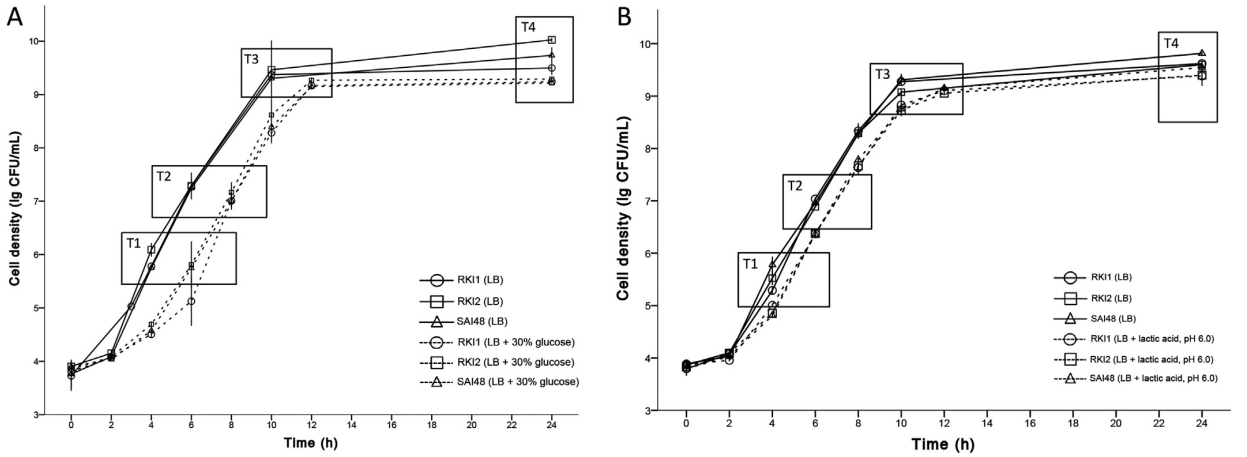

Fig. 1. Growth of three *S. aureus* strains (RKI1, RKI2, SAI48) was compared under no-stress conditions, under glucose stress, and under lactic acid stress. Cells were harvested in early exponential (T1), mid-exponential (T2), early stationary (T3), and late stationary growth phase (T4). 1A) LB and LB + 30% glucose; 1B) LB and LB + lactic acid (pH 6.0).

quantification data under glucose stress (*gyrB*, *ftsZ*, *pyk*) and two reference genes under lactic acid stress (*rho*, *rplD*).

3.2. Effect of glucose stress on growth and sed expression

Growth behavior of three *S. aureus* strains in LB and LB with 30% glucose is presented in Fig. 1A. Under glucose stress, the mean lag phase duration was prolonged compared with no-stress (Δ2.55 ± 0.47 h; $p = 0.00$), and maximum cell density was decreased in late stationary phase (Δ0.53 ± 0.22 lg CFU mL^{-1}; $p = 0.00$). However, the growth rate was slightly increased under glucose stress (Δ0.09 ± 0.01 lg CFU mL^{-1} h^{-1}; $p = 0.03$). Glucose stress had no statistically significant effect on *sed* expression during early (T1) and mid-exponential (T2) as well as early stationary (T3) growth phases. However, we observed a trend towards reduced *sed* expression during late stationary phase, with a statistically significant reduction of *sed* expression in one strain (RKI2, $p = 0.00$) (Table 3, Fig. 2).

3.3. Effect of lactic acid stress on growth and sed expression

Growth behavior of three *S. aureus* strains in LB with MES and LB with lactic acid (pH 6.0) is presented in Fig. 1B. Under lactic acid stress, the mean lag phase duration was prolonged (Δ0.76 ± 0.01 h; $p = 0.00$) and maximum cell density was decreased in late stationary phase (Δ0.34 ± 0.09 lg CFU mL^{-1}; $p = 0.00$). However, the growth rate remained unchanged under both growth conditions (Δ0.00 ± 0.05 lg CFU mL^{-1} h^{-1}; $p = 0.95$). All strains increased *sed* expression over time. Growth under lactic acid stress did not significantly alter *sed* expression (Table 4, Fig. 3).

3.4. Strain-specific differences in sed expression

Comparing *sed* expression between strains, the food poisoning strains RKI1 and RKI2 tended to exhibit higher relative *sed* expression ratios than strain SAI48 associated with a case of infection. When grown in LB only, *sed* expression was significantly higher during late stationary growth phase (T4) in RKI2 compared to SAI48 ($p < 0.05$). Under glucose stress, *sed* expression was significantly higher during early exponential phase (T1) in RKI1 ($p = 0.01$) and RKI2 ($p = 0.00$) compared with SAI48. Under lactic acid stress, *sed* expression was significantly higher during late stationary phase (T4) in RKI2 compared to SAI48 ($p = 0.048$).

4. Discussion

As staphylococcal enterotoxins are extremely stable and cannot be inactivated by measures such as heating of food, it is crucial to prevent enterotoxin formation by preventing *S. aureus* growth in the food matrix. Various stress conditions were suggested to inhibit staphylococcal growth, with glucose being reported to lead to an even more pronounced inhibition of *S. aureus* growth than high concentrations of salts (Vilhelmsson & Miller, 2002). Vilhelmsson and Miller linked catabolite repression and osmotic stress responses in *S. aureus*, predicting that the use of glucose and other metabolizable carbohydrates as humectants should result in a decreased growth rate in *S. aureus* (Vilhelmsson & Miller, 2002). In this study, glucose stress (30% glucose) and lactic acid stress (pH 6.0) resulted in a prolonged mean lag phase and a decreased maximum cell density in late stationary phase. However, the growth rate of the tested *S. aureus* strains was slightly increased under glucose stress. This is consistent with previous findings

Table 3
Effect of glucose stress on *sed* expression. Fold change of *sed* expression in three strains (RKI1, RKI2, SAI48) in early exponential (T1), mid-exponential (T2), early stationary (T3) and late stationary phase (T4) is indicated relative to the mean expression level at T1 in LB. Statistically significant changes in *sed* expression in LB + 30% glucose compared to LB only are marked by an asterisk.

Growth phase	LB			LB + 30% glucose		
	RKI1	RKI2	SAI48	RKI1	RKI2	SAI48
T1	1.0 (±0.3)	1.0 (±0.6)	1.0 (±0.9)	1.0 (±0.6)	0.9 (±0.5)	0.5 (±0.1)
T2	1.0 (±0.6)	1.1 (±0.8)	3.1 (±1.4)	1.3 (±0.3)	0.7 (±0.5)	2.4 (±1.2)
T3	2.0 (±1.0)	1.8 (±1.4)	2.8 (±1.1)	2.4 (±1.1)	2.0 (±1.3)	3.2 (±1.1)
T4	2.4 (±1.1)	3.6 (±2.3)	3.3 (±0.5)	0.9 (±0.3)	0.6 (±0.3)* ↓	2.5 (±1.6)

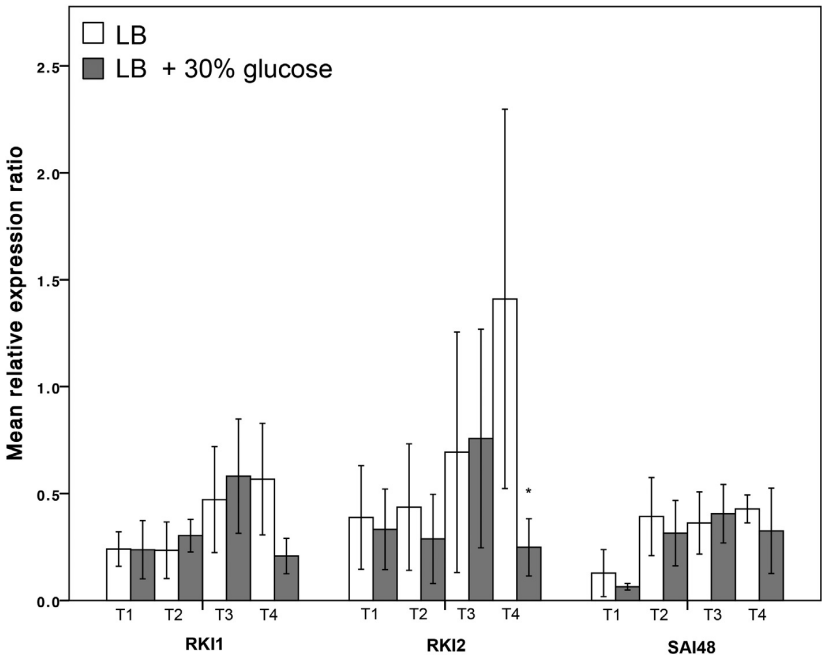

Fig. 2. Effect of glucose stress on *sed* expression in three *S. aureus* strains (RKI1, RKI2, SAI48) in early exponential (T1), mid-exponential (T2), early stationary (T3), and late stationary phase (T4). Statistically significant changes in *sed* expression in LB adjusted to 30% glucose compared to LB are marked by an asterisk ($p < 0.05$).

reporting an increased growth rate of *S. aureus* in the presence of glucose (Jarvis, Lawrence, & Pritchard, 1975).

Previous studies suggest that the effect of a low pH on *S. aureus* growth and enterotoxin formation varies with regard to the acid and strain tested (Domenech et al., 1992; Genigeorgis et al., 1971; Genigeorgis & Sadler, 1966; Rode et al., 2010; Rosengren, Lindblad, & Lindqvist, 2013; Wallin-Carlquist et al., 2010). In general, growth and enterotoxin formation were reported to subside at pH values below 4.0 in aerobically cultured cells and pH 5.0 in anaerobically cultured cells (Domenech et al., 1992; Smith, Buchanan, & Palumbo, 1983). *S. aureus* was shown to grow within the pH and lactic acid ranges characteristic for the milk fermentation and cheese making process at conditions simulating the initial stages of cheese production (Rosengren et al., 2013). In this study, we used lactic acid and pH 6.0 to mimic conditions encountered in many food matrices associated with staphylococcal food poisoning.

Weinrick et al. reported that a decline in pH leads to changes in gene expression including reduced enterotoxin expression formerly thought to represent a glucose effect (Weinrick et al., 2004; Jarvis et al., 1975). Since *S. aureus* represents one of the most osmotolerant foodborne pathogens, a glucose concentration of 30% was chosen to introduce osmotic stress and to mimic conditions in food products with high sugar content (*e.g.* cake or condensed milk). In our study, cells grown at 30% glucose exhibited significantly decreased *sed* expression in late stationary phase in strain RKI2, a trend towards decreased *sed* expression in RKI1, and unaltered *sed* expression in SAI48.

In our study, lactic acid stress had no statistically significant effect on *sed* expression. For enterotoxin SEA, it has been reported that mild lactic acid stress can increase the formation of SEA (Rosengren et al., 2013). This is of particular interest, as lactic acid was reported to effectively inhibit enterotoxin production (Domenech et al., 1992).

In our study, the two clinical strains associated with staphylococcal food poisoning were shown to express *sed* at higher levels compared to the *S. aureus* strain isolated from a case of infection. To date, there are no comprehensive studies demonstrating a link between *sed* expression level and the strain origin, in particular for strains isolated from food poisoning outbreaks. However, for SEB producing strains, host factors have been shown to have an impact on toxin levels produced (Compagnone-Post, Malyankar, & Khan, 1991). Moreover, higher enterotoxin C production levels have been linked to strains of human and food origin, in contrast to animal strains with lower SEC production (Marr et al., 1993).

In conclusion, our results indicate that both glucose and pH stress have an effect on *S. aureus* growth, with a prolongation of the lag phase and decreased maximum cell densities reached in late stationary phase. In addition, glucose stress slightly increased the

Table 4
Effect of lactic acid stress on *sed* expression. Fold change of *sed* expression in three strains (RKI1, RKI2, SAI48) in early exponential (T1), mid-exponential (T2), early stationary (T3) and late stationary phase (T4) is indicated relative to the mean expression level at T1 in LB with MES.

Growth phase	LB			LB + lactic acid (pH 6.0)		
	RKI1	RKI2	SAI48	RKI1	RKI2	SAI48
T1	1.0 (±0.3)	1.0 (±0.6)	1.0 (±0.6)	2.6 (±2.9)	1.1 (±0.9)	0.6 (±0.3)
T2	1.1 (±0.1)	0.8 (±0.4)	0.6 (±0.4)	5.9 (±8.7)	1.0 (±0.5)	10.3 (±11.6)
T3	4.7 (±5.1)	3.8 (±2.3)	3.7 (±3.4)	28.2 (±19.7)	8.3 (±3.3)	19.4 (±19.8)
T4	36.6 (±47.9)	16.0 (±4.3)	14.1 (±10.1)	39.1 (±13.2)	26.1 (±8.4)	28.5 (±11.2)

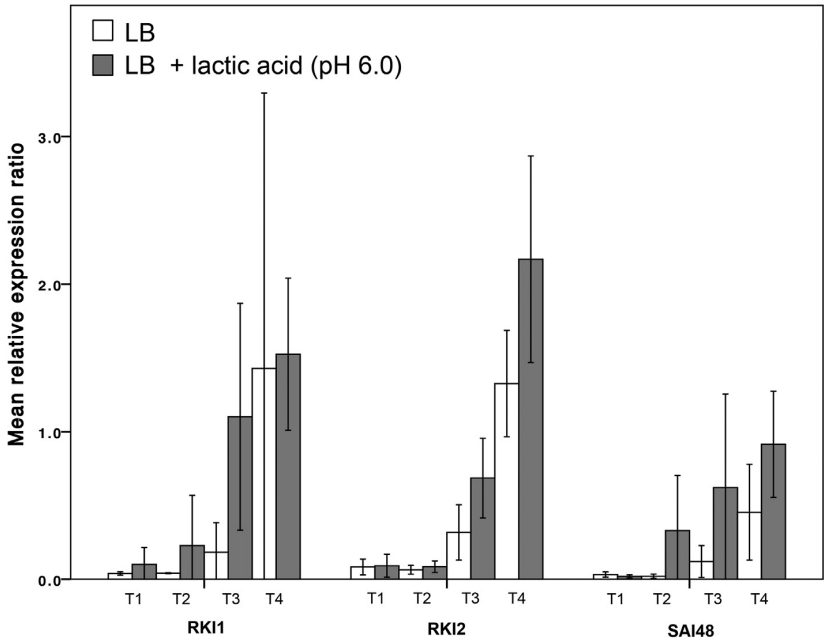

Fig. 3. Effect of lactic acid stress on *sed* expression in three *S. aureus* strains (RKI1, RKI2, SAI48) in early exponential (T1), mid-exponential (T2), early stationary (T3), and late stationary phase (T4).

growth rate of the *S. aureus* strains and led to decreased *sed* expression in late stationary phase. Lactic acid stress had no statistically significant effect on *sed* expression of the tested *S. aureus* strains. The data generated in this study specifies the effect of critical food-related stressors on growth and SE expression of *S. aureus,* which can be used for risk assessment.

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5.4 Publication 12

Effect of sodium nitrite and regulatory mutations Δ*agr*, Δ*sarA*, and Δ*sigB* on the mRNA and protein levels of staphylococcal enterotoxin D

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Effect of sodium nitrite and regulatory mutations Δ*agr*, Δ*sarA*, and Δ*sigB* on the mRNA and protein levels of staphylococcal enterotoxin D

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ABSTRACT

Staphylococcal food poisoning results from ingestion of enterotoxins produced by *Staphylococcus aureus*. Staphylococcal enterotoxin D (SED) is one of the most common toxins detected in *S. aureus* strains associated with intoxications. The effect of sodium nitrite on enterotoxin production has been only partly investigated, despite its wide usage in meat products. In addition, the factors influencing SED regulation are unclear. The aim of this study was to determine the effect of sodium nitrite on *sed* transcription and SED production, as well as the effect of regulatory mutations on SED protein levels. Temporal *sed* mRNA and SED protein levels were compared in LB and LB supplemented with 150 mg/L nitrite, and SED protein levels between wild type (wt) and isogenic regulatory mutants (Δ*agr*, Δ*sarA*, Δ*sigB*) under control and sodium nitrite conditions. Relative *sed* mRNA levels of wt strains were higher in late stationary phase in the presence of nitrite compared to control conditions. However, SED protein levels were decreased in the presence of nitrite. In LB, Δ*agr* mutants showed SED levels similar to the wt, while Δ*sarA* mutants exhibited reduced and Δ*sigB* mutants increased SED levels compared to the wt. In LB with sodium nitrite, SED levels of mutant strains were reduced similar to the wt strains, except for two Δ*agr* mutants, in which SED levels were increased in the presence of nitrite. Overall, strain-specific variation with regard to the effect of regulatory mutations was observed. In addition, the data suggests that SED regulation may not be as tightly dependent on Agr as previously described.

1. Introduction

Staphylococcus (S.) aureus can give rise to various diseases such as local and systemic infections and toxin-mediated diseases. Staphylococcal food poisoning is an intoxication caused by preformed staphylococcal enterotoxins in food. On average, 240,000 cases are estimated to occur yearly in the US (Scallan et al., 2011), and 3000 cases are reported yearly in the EU (European Food Safety Authority, 2015).

Sodium nitrite ($NaNO_2$) is a widely used food additive contributing to the preservation, red meat color, and cured flavor of various meat products such as bacon, ham, and sausages. The red color of the meat is retained when myoglobin and hemoglobin react with nitric oxide resulting from the reduction of nitrite. In *Clostridium botulinum*, sodium nitrite has been shown to inhibit growth by interfering with the formation of iron-sulfur clusters (Duncan & Foster, 1968; Pierson & Smoot, 1982; Reddy, Lancaster, & Cornforth, 1983). The desired cured meat flavor is obtained with relatively low levels of nitrite (50 mg/kg) (Mac Donald, Stanley, & Usborne, 1980). The mechanisms underlying its bactericidal and bacteriostatic action are not fully understood, but inhibition of oxygen uptake, uncoupling of oxidative phosphorylation, and inhibition of metabolic enzymes have been described (Tompkin, 2005).

Despite the wide utilization of sodium nitrite in food preservation, its effect on *Staphylococcus aureus* growth and enterotoxin gene expression has been only partially investigated and regulatory mechanisms controlling staphylococcal enterotoxin D (SED) production in the presence of sodium nitrite are unclear. Previous studies have shown that *S. aureus* growth is not affected by nitrite concentrations causing growth retardation in *C. botulinum* or *Listeria monocytogenes* (Buchanan & Solberg, 1972; Lövenklev, Holst,

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Borch, & Rådström, 2004; Nyachuba, Donnelly, & Howard, 2007). However, an influence of pH on growth inhibition by sodium nitrite has been demonstrated in several bacterial species (Castellani & Niven, 1955; Tarr, 1941). Sodium nitrite was shown to inhibit growth and production of staphylococcal enterotoxin A (SEA) at pH values below 7.0 (Tompkin, Ambrosino, & Stozek, 1973), which corresponds to pH levels encountered in most meat products supplemented with sodium nitrite. It has been reported that nitrite concentrations of up to 200 mg/L did not affect *S. aureus* growth or staphylococcal enterotoxin B (SEB) production (McLean, Lilly, & Alford, 1968). In contrast, in sausages supplemented with nitrite (c = 154 mg/kg), no SEA and SED formation was detected by ELISA despite *S. aureus* growth to 10^7 CFU/g (Bang, Hanson, & Drake, 2008).

Production of plasmid encoded SED is regulated by several regulatory elements including accessory gene regulator (Agr), staphylococcal accessory regulator (SarA), sigma factor B (SigB), and repressor of toxins (Rot). Agr is a two-component quorum sensing system activated by increased cell density. Upon activation, the transcription of cell wall-associated proteins is repressed and exotoxin transcription is increased (Bronner, Monteil, & Prévost, 2004). DNA binding protein SarA regulates virulence gene transcription via Agr-dependent and independent mechanisms (Chien, Manna, Projan, & Cheung, 1999) increasing expression of several exotoxins such as *seb* and *tst* (Chan & Foster, 1998). Alternative sigma factor SigB is activated post-translationally by several environmental stresses and functions antagonistically to Agr (Novick, 2003). Rot is a global regulator repressing transcription of several exotoxins (Tseng & Stewart, 2005; Tseng, Zhang, & Stewart, 2004). Most studies investigating the effect of regulatory mutations have however been conducted using derivatives of strain NCTC8325 harboring an 11-base deletion in *rsbU*, a gene encoding an indirect positive regulator of SigB (Gertz et al., 1999). Since a defect in the *sigB* operon has been shown to affect global regulators Agr, Sar, and Rot, results generated using NCTC8325 derivatives may not be representative (Bischoff, Entenza, & Giachino, 2001; Cassat et al., 2006; Hsieh, Tseng, & Stewart, 2008; Lauderdale, Boles, Cheung, & Horswill, 2009).

The aim of this study was to determine the effect of sodium nitrite on *sed* transcription and SED production, as well as the effect of regulatory mutations (*Δagr*, *ΔsarA*, *ΔsigB*) on SED protein levels in three different *S. aureus* strains originating from food poisoning outbreaks and an infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. aureus strains used in this study are listed in Table 1. Isogenic mutant strains were constructed by transduction using phage 80α as previously described (Charpentier et al., 2004; Sihto, Tasara, Stephan, & Johler, 2015). The strains were grown in Luria Bertani broth (LB, Difco laboratories, Detroit, MI) (Bertani, 1951) and in LB supplied with sodium nitrite ($NaNO_2$) (Pacovis AG, Stetten, Switzerland). Nitrite concentration of 150 mg/L (a_w = 0.98) was chosen to correspond to the maximum amount generally added in meat products in the EU (EC., 2011). The growth phases of all strains under control and sodium nitrite conditions were determined by viable cell counts using plate count agar (Sigma–Aldrich, Stockholm, Sweden), with incubation of the plates at 37 °C for 18–24 h.

Single colonies were transferred from 5% sheep blood agar to 5 mL of LB broth and grown for 18 h (37 °C, 225 rpm). Aliquots of 1 mL of the overnight cultures were centrifuged with an Eppendorf 5424 (6000 × g for 10 min) and washed twice with 0.8% NaCl (Merck, Darmstadt, Germany) to remove residual media components. LB and LB supplemented with sodium nitrite were inoculated with 10^{-3} dilution of washed overnight culture to result in approximate cell density of 5×10^3 CFU/mL and incubated at 37 °C, 225 rpm. For RKI2Δ*sarA*, the 10^{-2} dilution was used instead of the 10^{-3} dilution to account for an extended lag phase in this strain. Culture supernatant samples for ELISA were harvested by centrifugation (14,000 × g for 1 min) at 2 h intervals until 12 h, and after 24 h. Two independent cultivations were performed for all strains to gain two independent samples of each strain, condition, and time point.

Samples for RNA extraction were harvested by centrifugation (8000 × g for 5 min) in early exponential (T1), mid-exponential (T2), early stationary (T3), and late stationary phase (T4). Cell pellets were resuspended in 500 μl RNA protect bacteria reagent (Qiagen, Hombrechtikon, Switzerland), incubated at room temperature for 5 min and harvested again by centrifugation (3000 × g for 5 min). Cell pellets were stored at −80 °C for a duration of several hours to several weeks before being used for RNA extraction.

2.2. RNA extraction and reverse transcription

Cell lysis, RNA extraction, and reverse transcription were performed as previously described (Sihto, Tasara, Stephan, & Johler, 2014). For each sample, 100 ng of RNA was converted to cDNA. The reverse transcription reaction was performed twice for each sample. RNA integrity numbers were determined by Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and ranged from 7.1 to 9.1.

2.3. Quantitative real-time PCR (qPCR)

Single peaks in the melting curve analyses and single product

Table 1
S. aureus wt and mutant strains used in this study.

Strain ID	Relevant characteristics	Source (reference)
RKI1	strain associated with foodborne outbreak, CC8/t648, *sea*+, *sed*+, *sej*+	Robert Koch Institute, Germany
RKI2	strain associated with foodborne outbreak, CC8/t008, *sea*+, *sed*+, *sej*+	Robert Koch Institute, Germany
SAI48	strain isolated from *S. aureus* infection, CC5/t002, *sec*+, *sed*+, *sej*+	Institute of Medical Microbiology, University of Zurich, Switzerland
RKI1Δ*agr*	RKI1 with *ermB* replacing *agr*	(Sihto et al., 2014)
RKI1Δ*sigB*	RKI1 with *ermB* replacing *sigB*	(Sihto et al., 2014)
RKI1Δ*sarA*	RKI1 with *tetL* replacing *sarA*	(Sihto et al., 2014)
RKI2Δ*agr*	RKI2 with *ermB* replacing *agr*	(Sihto et al., 2014)
RKI2Δ*sigB*	RKI2 with *ermB* replacing *sigB*	(Sihto et al., 2014)
RKI2Δ*sarA*	RKI2 with *tetL* replacing *sarA*	(Sihto et al., 2014)
SAI48Δ*agr*	SAI48 with *ermB* replacing *agr*	(Sihto et al., 2014)
SAI48Δ*sigB*	SAI48 with *ermB* replacing *sigB*	(Sihto et al., 2014)
SAI48Δ*sarA*	SAI48 with *tetL* replacing *sarA*	(Sihto et al., 2014)

Table 2
Primer pairs, including amplicon sizes, primer concentrations, efficiency (E) values, and annealing temperatures for the target and reference genes used in this study.

Gene	Function	Primer pair (5′ - 3′)	Amplicon size (bp)	Primer conc. (nM)	E (%)	Annealing T (°C)	Reference
sed	Staphylococcal enterotoxin D	GTG GTG AAA TAG ATA GGA CTG C	384	250	90–98	62	(Monday & Bohach, 1999)
		ATA TGA AGG TGC TCT GTG G		250			
rho	Transcription termination factor	GAA GCT GCT GAA GTC G	319	250	97–98	57	(Sihto et al., 2014)
		CGT CCA TAC GTG AAC CC		250			
gyrB	DNA gyrase (subunit B)	GTC GAA GGG GAC TCT G	242	250	91–96	57	(Sihto et al., 2014)
		GCT CCA TCC ACA TCG G		250			
proC	Pyrroline-5-carboxylate reductase	GGC AGG TAT TCC GAT TG	231	800	93–96	57	(Sihto et al., 2014)
		CTT CCG GTG ATA GCT GTT A		1000			

bands on agarose gels confirmed target-specific amplifications for all primers (Table 2). qPCR experiments were performed using a Light-Cycler-480 (Roche). A total reaction volume of 10 μl was used, including 4 μl cDNA template (dilution 1:100), optimized concentration of each primer, and the LightCycler480 SYBR Green I master mix (Roche). Water (no template) and RT minus (no reverse transcriptase) samples were used as controls. An inter-run calibrator sample was included as a control for variation in cDNA synthesis and amplification. PCR cycling conditions included 8 min at 95 °C, 45 amplification cycles (95 °C for 10 s, the respective annealing temperature for 15 s, 72 °C for 20 s, 78 °C for 1 s with a single fluorescence measurement), a melting curve (60–95 °C at 2.2 °C/s and a continuous fluorescence measurement), and a final cooling step. Standard curves based on genomic DNA were generated to determine the efficiency of target gene amplification for each strain. All samples were amplified in triplicate. Expression levels of *sed* were normalized using *rho, gyrB*, and *proC* reference genes selected as previously described (Sihto et al., 2014). The effect of sodium nitrite on *sed* expression was assessed by comparing relative expression ratios between control and sodium nitrite conditions at the same time point in early exponential, mid-exponential, early stationary, and late stationary phases.

2.4. ELISA

ELISA analysis of SED was performed according to the protocol for SEA as previously described (Wallin-Carlquist et al., 2010) with minor modifications: Instead of SEA antibodies, SED affinity-purified sheep polyclonal antibodies (Toxin Technology Inc., Sarasota, FL) were used. Each sample was analyzed in three technical replicates. The reliability of the technical replications was verified using Cronbach's Alpha (0.990).

2.5. Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (SPSS Inc., Chicago, IL). Results were considered significant at $p < 0.05$. Growth parameters such as exponential phase growth rate and maximum cell density were determined using DMFit 3.0 (Baranyi & Roberts, 1994) and compared using Student's t-test. For qPCR data, log transformed relative expression ratios were compared using one-way ANOVA. For ELISA data, log transformed SED levels between different growth conditions (LB or LB supplemented with sodium nitrite) were compared using three-way mixed design ANOVA. SED levels in wild type (wt) and isogenic regulatory mutants were compared using one-way ANOVA.

3. Results

3.1. Impact of sodium nitrite on growth, SED production, and sed transcription in S. aureus wt strains

Growth behavior and SED production of *S. aureus* strains RKI1, RKI2, and SAI48, in LB and LB supplemented with 150 mg/L nitrite are presented in Fig. 1. Growth rates (Δ0.07 ± 0.08 log CFU mL^{-1} h^{-1}; $p = 0.44$) and maximum cell densities in stationary phase (Δ0.20 ± 0.60 log CFU/mL; $p = 0.54$) between the strains were similar under both growth conditions.

SED was detectable by ELISA after 6 h of incubation at a cell density of around 10^6 CFU/mL (Fig. 1). SED levels gradually

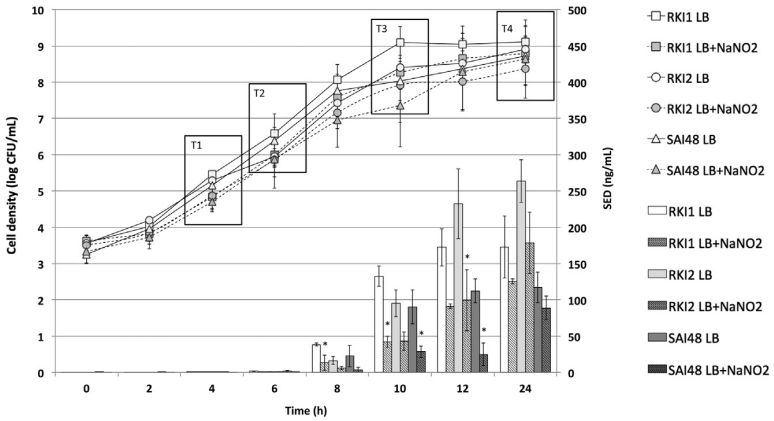

Fig. 1. Growth and SED production of three wild type *S. aureus* strains (RKI1, RKI2, SAI48) in LB and LB supplemented with nitrite (150 mg/L). Cell harvesting time points for RNA extraction are designated as T1, T2, T3, and T4. Error bars represent one standard deviation of the mean (n = 2). Statistically significant changes in SED production between LB and LB + $NaNO_2$ at the same time point are marked by an asterisk ($p < 0.05$).

Table 3
Three-way mixed design analysis of variance (ANOVA) identified a significant main effect of sodium nitrite on SED levels produced [$F(1, 9) = 68.933$, $p = 0.00$] and a significant interaction for strain × time × growth condition [$F(44, 36) = 6.597$, $p = 0.00$]. Statistically significant mean differences of log-transformed values between LB and LB supplemented with nitrite (150 mg/L) are marked by an asterisk.

Strain	Time (h)	Growth condition	Mean difference (LB − LB + NaNO2)	Mean	95% Confidence interval	
					Lower bound	Upper bound
RKI1 wt	6	LB	0.024*	1.374	1.236	1.511
		LB + $NaNO_2$		1.350	1.212	1.487
	8	LB	0.330	1.781	1.644	1.918
		LB + $NaNO_2$		1.451	1.313	1.588
	10	LB	0.381*	2.189	2.051	2.326
		LB + $NaNO_2$		1.808	1.671	1.945
	12	LB	0.216	2.287	2.149	2.424
		LB + $NaNO_2$		2.070	1.933	2.208
	24	LB	−0.013	2.285	2.147	2.422
		LB + $NaNO_2$		2.298	2.160	2.435
RKI1Δ*agr*	6	LB	−0.003	1.358	1.221	1.496
		LB + $NaNO_2$		1.361	1.224	1.499
	8	LB	−0.002	1.602	1.465	1.739
		LB + $NaNO_2$		1.604	1.467	1.741
	10	LB	−0.228	2.100	1.963	2.237
		LB + $NaNO_2$		2.328	2.134	2.522
	12	LB	−0.102	2.236	2.099	2.374
		LB + $NaNO_2$		2.338	2.200	2.475
	24	LB	−0.150	2.311	2.173	2.448
		LB + $NaNO_2$		2.460	2.323	2.598
RKI1Δ*sarA*	6	LB	0.007	1.349	1.212	1.486
		LB + $NaNO_2$		1.342	1.205	1.480
	8	LB	0.182	1.540	1.403	1.677
		LB + $NaNO_2$		1.358	1.221	1.495
	10	LB	0.430*	1.982	1.844	2.119
		LB + $NaNO_2$		1.552	1.414	1.689
	12	LB	0.320*	2.143	2.006	2.280
		LB + $NaNO_2$		1.823	1.685	1.960
	24	LB	0.138	2.135	1.997	2.272
		LB + $NaNO_2$		1.997	1.859	2.134
RKI1Δ*sigB*	6	LB	0.007	1.385	1.248	1.523
		LB + $NaNO_2$		1.378	1.240	1.515
	8	LB	0.012	1.614	1.476	1.751
		LB + $NaNO_2$		1.601	1.464	1.739
	10	LB	−0.062	2.098	1.960	2.235
		LB + $NaNO_2$		2.160	1.965	2.354
	12	LB	0.204	2.450	2.313	2.587
		LB + $NaNO_2$		2.246	2.108	2.383
	24	LB	0.125	2.639	2.502	2.776
		LB + $NaNO_2$		2.514	2.377	2.651
RKI2 wt	6	LB	0.014	1.364	1.226	1.501
		LB + $NaNO_2$		1.350	1.212	1.487
	8	LB	0.133	1.583	1.446	1.721
		LB + $NaNO_2$		1.451	1.313	1.588
	10	LB	0.259	2.067	1.929	2.204
		LB + $NaNO_2$		1.808	1.671	1.945
	12	LB	0.331*	2.402	2.264	2.539
		LB + $NaNO_2$		2.070	1.933	2.208
	24	LB	0.157	2.455	2.317	2.592
		LB + $NaNO_2$		2.298	2.160	2.435
RKI2Δ*agr*	6	LB	0.040	1.383	1.246	1.520
		LB + $NaNO_2$		1.343	1.205	1.480
	8	LB	0.277*	1.646	1.508	1.783
		LB + $NaNO_2$		1.368	1.231	1.506
	10	LB	0.487*	2.171	2.034	2.309
		LB + $NaNO_2$		1.684	1.546	1.821
	12	LB	0.482*	2.485	2.348	2.622
		LB + $NaNO_2$		2.003	1.866	2.141
	24	LB	0.285*	2.637	2.499	2.774
		LB + $NaNO_2$		2.352	2.215	2.490
RKI2Δ*sarA*	6	LB	0.031	1.379	1.242	1.516
		LB + $NaNO_2$		1.348	1.211	1.486
	8	LB	0.223	1.608	1.471	1.746
		LB + $NaNO_2$		1.385	1.248	1.522
	10	LB	0.663*	2.097	1.960	2.235
		LB + $NaNO_2$		1.435	1.297	1.572
	12	LB	0.639*	2.436	2.299	2.574
		LB + $NaNO_2$		1.797	1.603	1.991
	24	LB	0.321*	2.532	2.394	2.669
		LB + $NaNO_2$		2.211	2.074	2.348
RKI2Δ*sigB*	6	LB	0.035	1.376	1.239	1.514

Table 3 (*continued*)

Strain	Time (h)	Growth condition	Mean difference (LB − LB + NaNO2)	Mean	95% Confidence interval	
					Lower bound	Upper bound
		LB + $NaNO_2$		1.342	1.204	1.479
	8	LB	0.381*	1.706	1.568	1.843
		LB + $NaNO_2$		1.325	1.187	1.462
	10	LB	1.073*	2.204	2.067	2.342
		LB + $NaNO_2$		1.131	0.994	1.269
	12	LB	0.523*	2.345	2.208	2.483
		LB + $NaNO_2$		1.823	1.685	1.960
	24	LB	−0.035	2.440	2.303	2.577
		LB + $NaNO_2$		2.475	2.338	2.613
SAI48 wt	6	LB	0.026	1.370	1.233	1.507
		LB + $NaNO_2$		1.344	1.207	1.481
	8	LB	0.232	1.638	1.500	1.775
		LB + $NaNO_2$		1.405	1.268	1.543
	10	LB	0.345*	2.046	1.909	2.183
		LB + $NaNO_2$		1.701	1.564	1.839
	12	LB	0.467*	2.127	1.989	2.264
		LB + $NaNO_2$		1.660	1.523	1.797
	24	LB	0.099	2.141	2.003	2.278
		LB + $NaNO_2$		2.042	1.905	2.180
SAI48Δ*agr*	6	LB	0.008	1.359	1.222	1.497
		LB + $NaNO_2$		1.351	1.214	1.488
	8	LB	0.106	1.620	1.483	1.757
		LB + $NaNO_2$		1.514	1.377	1.652
	10	LB	0.076	2.007	1.870	2.145
		LB + $NaNO_2$		1.931	1.794	2.069
	12	LB	−0.130	2.109	1.972	2.247
		LB + $NaNO_2$		2.239	2.102	2.377
	24	LB	−0.193	2.182	2.044	2.319
		LB + $NaNO_2$		2.374	2.237	2.512
SAI48Δ*sarA*	6	LB	0.026	1.370	1.233	1.508
		LB + $NaNO_2$		1.345	1.207	1.482
	8	LB	0.070	1.449	1.311	1.586
		LB + $NaNO_2$		1.379	1.241	1.516
	10	LB	0.226	1.812	1.674	1.949
		LB + $NaNO_2$		1.586	1.448	1.723
	12	LB	−0.024	2.035	1.897	2.172
		LB + $NaNO_2$		2.059	1.922	2.196
	24	LB	−0.261	2.024	1.887	2.161
		LB + $NaNO_2$		2.285	2.148	2.423
SAI48Δ*sigB*	6	LB	0.055	1.399	1.261	1.536
		LB + $NaNO_2$		1.344	1.207	1.482
	8	LB	0.387*	1.791	1.654	1.928
		LB + $NaNO_2$		1.404	1.267	1.542
	10	LB	0.597*	2.297	2.160	2.435
		LB + $NaNO_2$		1.700	1.563	1.837
	12	LB	0.611*	2.519	2.382	2.657
		LB + $NaNO_2$		1.908	1.771	2.046
	24	LB	0.236	2.584	2.446	2.721
		LB + $NaNO_2$		2.348	2.211	2.486

increased over time with the highest percent increase detected between 8 and 10 h, corresponding to late exponential growth phase.

Under control conditions, the highest SED level detected was reached during the stationary phase of growth in the three examined strains (at 12 h–24 h). RKI2 exhibited the highest SED levels, followed by RKI1 and SAI48. SED protein levels were generally lower in the presence of sodium nitrite compared to control conditions. The reduction was most pronounced between 8 and 12 h in all three strains and statistically significant in RKI1 (at 8 h and 10 h), RKI2 (at 12 h), and SAI48 (at 10 h and 12 h). Three-way mixed design ANOVA identified a significant main effect of sodium nitrite on SED levels produced [$F(1, 9) = 68.933$, $p = 0.00$] and a significant interaction for strain × time × growth condition [$F(44, 36) = 6.597$, $p = 0.00$] as shown in Table 3.

Temporal relative *sed* expression levels of strains RKI1, RKI2, and SAI48 generally increased from early exponential phase to late stationary phase under control conditions and in the presence of sodium nitrite (Fig. 2). Strain-specific differences in *sed* expression levels were observed, with RKI2 generally exhibiting higher *sed* expression levels, similar /to the SED levels determined by ELISA. Relative *sed* expression in RKI2 was statistically significantly higher compared to SAI48 ($p = 0.01$) and RKI1 ($p = 0.00$) under control conditions in late stationary phase (T4). Similarly, under sodium nitrite conditions RKI2 showed statistically significantly higher *sed* expression levels compared to SAI48 in early exponential and mid-exponential phase (T1-T2, $p < 0.02$). A tendency towards induced *sed* expression under sodium nitrite conditions was observed in all strains from mid-exponential to late stationary phase (T2-T4). In two strains (RKI1, SAI48), *sed* expression was significantly higher in T4 in the presence of sodium nitrite compared to control conditions ($p < 0.04$), in contrast to the reduced SED protein levels in the

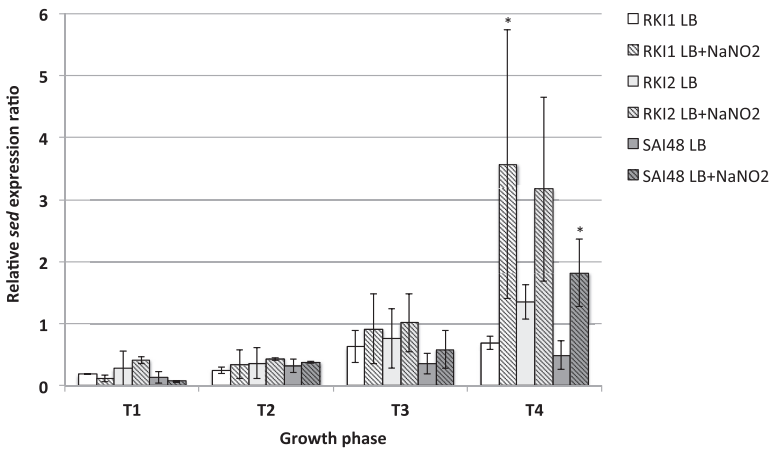

Fig. 2. Relative *sed* expression ratios of three wild type *S. aureus* strains (RKI1, RKI2, SAI48) in early exponential (T1), mid-exponential (T2), early stationary (T3), and late stationary phase (T4) in LB and in LB supplemented with nitrite (150 mg/L). Expression ratios are represented relative to the calibrator sample. Error bars represent one standard deviation of the mean (n = 4). Statistically significant changes in *sed* expression between LB and LB + $NaNO_2$ at the same time point are marked by an asterisk ($p < 0.05$).

presence of sodium nitrite determined by ELISA.

3.2. Impact of regulatory mutations on growth and SED production in LB and LB with sodium nitrite

Regulatory mutant strains grew similar to their parental wt strains under control and sodium nitrite conditions (Fig. 3). In addition, SED levels gradually increased over time. When grown in LB, all three *Δagr* mutants exhibited SED production levels similar to isogenic wt strains throughout all growth phases. In *ΔsarA* mutants of SAI48 and RKI1, SED levels were decreased compared to the wt ($p = 0.03$ at 8 h), while loss of SarA showed no effect in RKI2. In *ΔsigB* mutants of SAI48 and RKI1, SED levels were higher in early and late stationary phase. The increase in SED levels was statistically significant in *ΔsigB* mutants at 24 h ($p = 0.03$) (Table 4). At 24 h, SED levels were 3.1-fold higher in SAI48*ΔsigB* and 2.5-fold higher in RKI1*ΔsigB* compared to their respective wt strains. In contrast, SED levels of RKI2*ΔsigB* remained in the same level as in wt.

In regulatory mutants, SED production was reduced under sodium nitrite conditions similar to wt strains (Fig. 3, Table 3). However, in SAI48*Δagr* and RKI1*Δagr*, a trend towards increased SED production under sodium nitrite conditions was observed.

4. Discussion

Nitrate and nitrite are widely used in meat, fish, and cheese products to inhibit bacterial growth, maintain the color of the meat, and create the cured flavor. In this study, the effect of sodium nitrite and regulatory mutations on *sed* expression was determined in three *S. aureus* strains and their isogenic regulatory mutants. Using the maximum average concentration of nitrite in meat products, the growth rate and maximum cell density of the *S. aureus* strains were only slightly affected. This observation is in agreement with previous studies showing no growth inhibition by nitrite in concentrations <200 mg/L (Bang et al., 2008; Buchanan & Solberg, 1972). While sodium nitrite had only a modest effect on growth, *sed* mRNA and SED protein levels were notably affected. On the transcriptional level, relative *sed* mRNA levels were significantly increased in the presence of sodium nitrite in late stationary phase compared to the control conditions in two out of three strains ($p \leq 0.01$). On the protein level, SED production was nonetheless decreased in wt strains and most regulatory mutants in the presence of sodium nitrite in late exponential and early stationary growth phase. These findings demonstrate that *sed* transcription levels do not always reflect extracellular SED protein levels as previously shown for *sea* and *sec* (Valihrach, Alibayov, Zdenkova, & Demnerova, 2014; Zeaki, Cao, Skandamis, Rådström, & Schelin, 2014; Zeaki, Rådström, & Schelin, 2015).

Interestingly, none of the Δ*agr* mutants tested in this study showed reduced SED production compared to its isogenic wt. Agr is the most studied regulatory element in *S. aureus* and has been regarded as one of the main positive regulators involved in the expression of several enterotoxins including SED (Thoendel, Kavanaugh, Flack, & Horswill, 2011). More recent studies indicate however that the importance of Agr may have been overestimated due to the use of SigB deficient derivatives of strain NCTC8325 (Cassat et al., 2006; Schmidt, Donegan, Kwan, & Cheung, 2004). The lack of SigB activity appears to result in increased RNAIII expression and subsequent overactivation of the *agr* system (Lauderdale et al., 2009). Previous studies showing decreased production of SEB, SEC, and SED in Δ*agr* mutants (Bayles & Iandolo, 1989; Gaskill & Khan, 1988; Regassa, Couch, & Betley, 1991) were conducted using a strain designated as ISP546 (Mallonee, Glatz, & Pattee, 1982) representing a derivative of NCTC8325. This notion is also consistent with Schmidt et al. (Schmidt et al., 2004) suggesting that Agr is an inducer of *seb* only if the *sigB* operon is not functional, based on the contradictory effect of Δ*agr* mutation in NCTC8325 and the Newman strain with an intact *sigB* operon. The post-exponential increase in *sed* transcription has been reported to indirectly result from reduction of Rot activity by the Agr system rather than from a direct effect of Agr (Tseng et al., 2004).

Decreased *sed* promoter activity in Δ*sarA* mutants has been previously reported by Tseng et al. (Tseng et al., 2004). In this study, a tendency towards decreased SED levels in two out of three Δ*sarA* mutants was observed, with a statistically significant reduction in late exponential growth phase. For SEB, Chan et al. showed reduced protein levels of the enterotoxin D in Δ*sarA*, Δ*agr*, and Δ*agr*Δ*sarA* mutants (Chan & Foster, 1998).

In this study, two of three Δ*sigB* mutants exhibited statistically significantly increased SED levels in late stationary phase. This is in agreement with previous studies reporting increased *seb* and *sed* promoter activity in Δ*sigB* mutants of parental strains harboring an intact *sigB* operon (Schmidt et al., 2004; Tseng et al., 2004). Schmidt

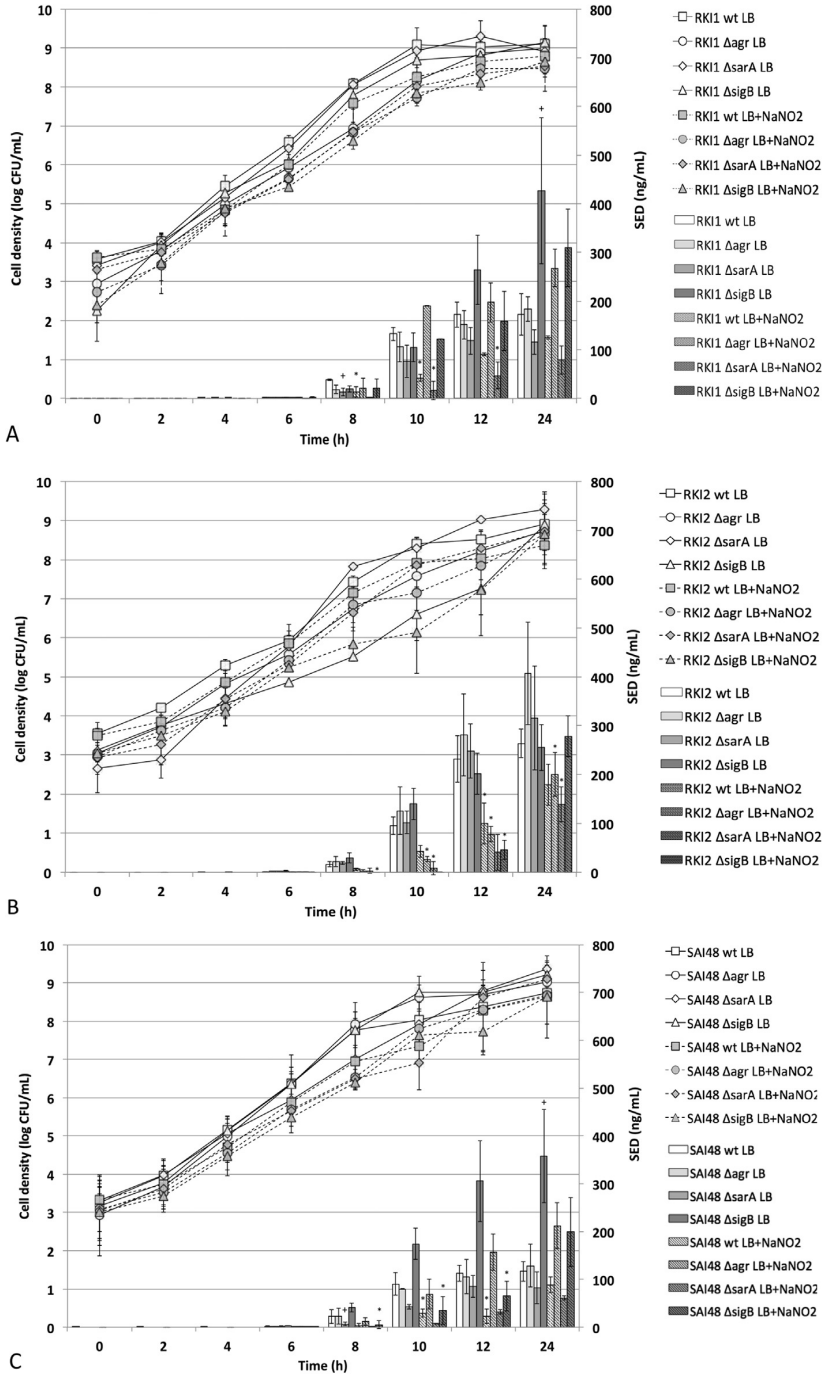

Fig. 3. Growth and SED production of three wild type *S. aureus* strains (RKI1, RKI2, SAI48) and their isogenic regulatory mutants (Δ*agr*, Δ*sarA*, Δ*sigB*) in LB and LB supplemented with nitrite (150 mg/L). Error bars represent one standard deviation of the mean (n = 2). Statistically significant changes in SED production between wt and isogenic mutant at the same time point and condition are marked by a plus symbol (+) and statistically significant changes in SED production between LB and LB + $NaNO_2$ at the same time point are marked by an asterisk (*) ($p < 0.05$). A) RKI1; B) RKI2; C) SAI48.

et al. (Schmidt et al., 2004) also demonstrated that the effect of loss of SigB is less pronounced in derivatives of NCTC8325 compared to the Newman strain. For SEB, increased production in a Δ*sigB* mutant of *S. aureus* strain COL has been demonstrated on the protein level (Ziebandt et al., 2001).

Interestingly, strain RKI2 generally deviated from the other two tested strains in terms of regulation of SED production. It was hypothesized that this may be due to sequence variation, but

Table 4
Significance values of one-way analysis of variance (ANOVA). SED levels were compared between wild type strains and isogenic regulatory mutants at time points 6–24 h in LB. Statistically significant differences are marked by an asterisk.

Time (h)	Mutation	p-value
6	Δ*agr*	0.801
	Δ*sarA*	0.750
	Δ*sigB*	0.065
8	Δ*agr*	0.444
	Δ*sarA*	0.029*
	Δ*sigB*	0.536
10	Δ*agr*	0.913
	Δ*sarA*	0.055
	Δ*sigB*	0.155
12	Δ*agr*	0.956
	Δ*sarA*	0.483
	Δ*sigB*	0.091
24	Δ*agr*	0.473
	Δ*sarA*	0.583
	Δ*sigB*	0.033*

sequencing of *sed* genes and *sed* promoter regions of the examined strains did not reveal any sequence variation related to differences in SED regulation (data not shown).

In this study, it was shown that sodium nitrite had a significant effect on *sed* transcription and SED production using a nitrite concentration of 150 mg/L. The reason(s) for such differences in transcription and production levels should be investigated further. The described findings also suggest strain-specific differences in *S. aureus* enterotoxin gene regulation. This underlines the findings of other studies demonstrating the importance of a multiple strain investigation approach when studying regulatory elements (Blevins, Beenken, Elasri, Hurlburt, & Smeltzer, 2002; Cassat et al., 2006; Nagarajan, Smeltzer, & Elasri, 2009). This is the first study investigating the effect of regulatory mutations (Δ*agr*, Δ*sarA*, Δ*sigB)* on SED mRNA and protein levels in several *S. aureus* strains other than derivatives of strain NCTC8325.

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6 Outbreak investigations and product-related studies

6.1 Publication 13

Outbreak of staphylococcal food poisoning due to SEA-producing *Staphylococcus aureus*

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Original Article

Outbreak of Staphylococcal Food Poisoning Due to SEA-Producing *Staphylococcus aureus*

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Abstract

In 2008, 150 people gathered for a wedding celebration in Baden-Württemberg, Germany. Three hours after ingestion of a variety of foods including pancakes filled with minced chicken, several guests exhibited symptoms of acute gastroenteritis such as vomiting, diarrhea, fever, and ague. Twelve guests were reported to have fallen ill, with nine of these seeking medical care in hospitals. At least four patients were admitted to the hospital and received inpatient treatment, among them a 2-year-old child and a woman in the 4th month of pregnancy. Within 24 h of the event, an investigative team collected a variety of samples including refrigerated leftovers, food in the storage unit of the caterer, nasal swabs of the caterer, as well as 21 environmental swabs. Five stool samples from patients were provided by the hospitals. *Staphylococcus aureus* isolates were gathered from eight samples, among them nasal swabs of the caterer, food samples, and one stool sample. Fourier transform–infrared spectroscopy was used for species identification and for primary clustering of the isolates in a similarity tree. The isolates were further characterized by *spa* typing and pulsed-field gel electrophoresis, and a DNA microarray was used to determine the presence/absence of genes involved in virulence and antimicrobial resistance. We were able to match an enterotoxigenic strain from the stool sample of a patient to isolates of the same strain obtained from food and the nasal cavity of a food handler. The strain produced the enterotoxin SEA and the toxic shock syndrome toxin-1, and was also found to exhibit the genes encoding enterotoxins SEG and SEI, as well as the enterotoxin gene cluster *egc*. This is one of only a few studies that were able to link a staphylococcal food poisoning outbreak to its source.

Introduction

While *Staphylococcus (S.) aureus* represents an ubiquitous commensal that persistently colonizes the anterior nares of 20–30% of the global population (van Belkum *et al.*, 2009), it can also cause severe infections, toxinoses, and life-threatening illnesses in humans and animals. Staphylococcal food poisoning is one of the most prevalent causes of foodborne intoxication worldwide. It is typically self-limiting, presenting with violent vomiting following a short incubation period. Within 2–6 h after ingestion of food containing staphylococcal enterotoxins (SEs), symptoms of acute gastroenteritis can be observed (Tranter *et al.*, 1990).

A variety of SEs and enterotoxin-like superantigens produced by *S. aureus* has been described, but only some were demonstrated to elicit an emetic response in a monkey-feeding assay. SEs that were shown to exhibit emetic activity include the classical enterotoxins SEA/SEB/SEC/SED/SEE, as well as the newly described enterotoxins SEG/SEH/SEI/SEJ (Thomas *et al.*, 2007). Although the toxic shock syndrome toxin-1 (TSST-1) shares many biological properties of SEs, it displays no emetic activity. However, TSST-1 can induce massive proliferation of T-cells and production of cytokines, leading to multisystem failure and lethal shock (Thomas *et al.*, 2007).

As clinical symptoms of staphylococcal food poisoning are often self-limiting, only 10% of patients with staphylococcal food poisoning are estimated to visit a hospital (Holmberg *et al.*, 1984). In a few of these patients, *S. aureus* can be isolated from a stool sample, and in an even far lower number of cases, the isolated strain can be matched to isolates collected from foodstuff, environmental samples, or food handlers. In many outbreak reports, stool samples are missing or negative, thus forcing the authors to try to evaluate the relevance of numerous enterotoxigenic staphylococcal isolates collected from different sources (Do Carmo *et al.*, 2004; Colombari *et al.*, 2007; Schmid *et al.*, 2009).

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In this study, we aim to (1) provide a comprehensive overview of an outbreak of staphylococcal food poisoning, and (2) identify and characterize the *S. aureus* strain responsible for the outbreak.

Outbreak Report

On June 21, 2008, 150 people gathered for a wedding celebration in Baden-Württemberg, Southwest Germany. Food was served at 4 p.m., with a caterer providing a buffet of pancakes filled with minced chicken, as well as a variety of meat (chicken, beef, pork), spaetzle, salad, soup, and cake. The wedding party contributed a watermelon.

Three hours later, the first guests complained about nausea and abdominal pain that progressed quickly to emesis with or without additional symptoms such as diarrhea, fever (39–40°C), cardiovascular problems, ague, and aching limbs. A total of 12 guests (8%) were reported to have fallen ill the night of June 21, with nine of these (6%) seeking medical care in local hospitals. While the youngest of them was only 2 years of age, the oldest patient was 53 years old. At least four patients (3% of the guests) were admitted to the hospital and received inpatient treatment over a period of 1–2 days, among them the 2-year-old child and a woman in the 4th month of pregnancy. All patients were diagnosed with gastroenteritis due to a presumptive food intoxication and were treated symptomatically by administration of intravenous fluids. As the general condition of one patient deteriorated on the second day, the patient was additionally treated with metamizole and metoclopramide. All patients recovered within 72 h, and no sequelae were reported. Eight of the nine patients who visited a hospital provided detailed anamnestic information to the investigative team by filling out a questionnaire.

Materials and Methods

Bacterial isolation and identification

Samples were taken on the day of the celebration and the day after, including refrigerated leftovers, food samples obtained from the storage unit of the caterer, nasal swabs of both nasal cavities of the caterer, as well as 21 environmental swabs. The hospitals provided stool samples of five patients for further investigation.

S. aureus were isolated from food samples using methods in accordance with §64 of the German Food and Feed Code (Amtliche Sammlung, L06.00-16 and L00.00-55). This corresponds to enumeration of coagulase-positive staphylococci by direct plating of decimal dilutions as described in EN ISO 6888-1: "Horizontal method for enumeration of coagulase-positive staphylococci." Coagulase-positive *Staphylococci* were identified by inoculating KRANEP agar (Oxoid, Wesel, Germany). Environmental swabs were streaked directly onto the selective agar plate that was subsequently incubated under aerobic conditions for 48 h at 37°C. RapiDEC staph® (bioMérieux, Nürtingen, Germany) was used to detect aurease production, and *S. aureus* species identification was confirmed using the latex agglutination test Staphytect Plus® (Oxoid). Fecal samples were fractionated on mannitol salt agar (MSA) and incubated at 37°C for 24 h. Subcultures of yellow colonies were used to inoculate Columbia blood agar (bioMérieux) that was incubated for 24 h at 37°C. Presumptive *S. aureus* isolates were confirmed by VITEK GP ID card (bioMérieux).

Fourier transform infrared spectroscopy (FT-IR)

FT-IR allowed for rapid species identification of *S. aureus* isolates (Spohr *et al.*, 2011). For this purpose, bacterial isolates were cultivated on sheep blood agar plates (Oxoid) at 37°C for 24 h. Sample preparation, FT-IR spectroscopy (using IFS 28/B spectrometer, BrukerOptics, Ettlingen, Germany) and data acquisition were performed as described previously (Kuhm *et al.*, 2009). Analysis of data was carried out using OPUS Software (vers. 4.2, BrukerOptics) and an artificial neural network built by the NeuroDeveloper software (Synthon, Heidelberg, Germany). The differentiation of *S. aureus* was performed with a method described previously (Spohr *et al.*, 2011).

IR spectra of isolates were compared by cluster analysis (Amiali *et al.*, 2007; Stamm *et al.*, 2013). For cluster analysis, the vector-normalized spectra of the wave number range 500–1600 cm^{-1} in second derivation were used for calculation with Ward's algorithm (OPUS 4.2; Ward *et al.*, 1963). The dendrogram obtained shows the arrangement of isolates in groups according to their spectral differences (Fig. 1).

Spa typing

The sequence of the polymorphic X region of the *spa* gene of the eight *S. aureus* isolates was determined as previously described (Aires de Sousa *et al.*, 2006), with minor modifications. Total DNA was isolated using the Qiagen DNeasy kit (Hilden, Germany), following the manufacturer's instructions. The *spa* gene was amplified as previously described (Wattinger *et al.*, 2012). Amplicons were purified and the concentration of nucleic acids was determined using a Nanodrop ND-UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). Sequencing was outsourced (Microsynth, Balgach, Switzerland). The sequences were assigned to *spa* types using the spa-server (http://www.spaserver.ridom.de/).

Microarray-based genotyping

The StaphyType ArrayStrip platform was used for DNA microarray profiling according to the manufacturer's instructions (Clondiag chip technologies, Jena, Germany). Similar to Coombs *et al.* (Coombs *et al.*, 2010), microarray profiles were compared using SplitsTree4 (http://www.splitstree.org).

PFGE

Preparation of chromosomal DNA and PFGE analysis of *Sma*I digested fragments was performed as previously described (Bannerman *et al.*, 1995). Electrophoresis was carried out in a CHEF-DR III electrophoresis cell (Bio-Rad). *Salmonella* serotype Braenderup strain H9812 digested with *Xba*I (50 U, 12 h, 37°C) was used as a molecular size standard.

RPLA toxin detection

S. aureus isolates collected from food samples were examined for production of superantigenic toxins including enterotoxins SEA/SEB/SEC/SED, as well as TSST-1 (toxic

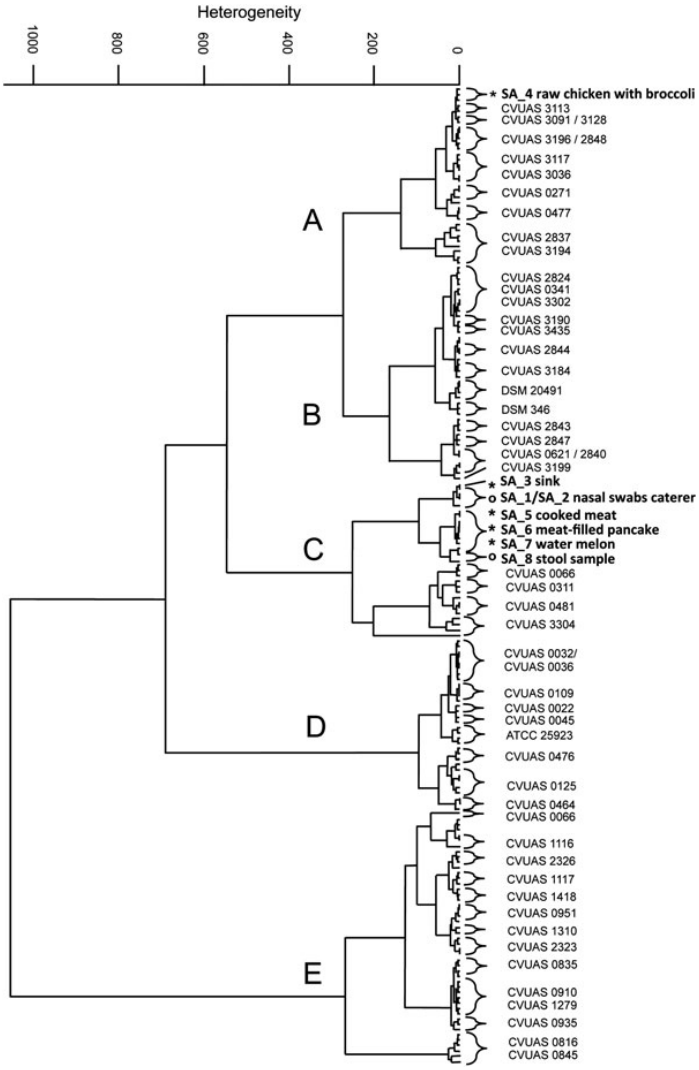

FIG. 1. Similarity tree depicting the results of the Fourier transform infrared spectroscopy analysis. Seven isolates (SA_1-3 and SA_5-8) formed a cluster of closely related isolates, while spectra of SA_4 were found on a different branch.

shock syndrome toxin-1) using RPLA toxin detection kits (Oxoid).

Results

S. aureus was isolated from eight different samples (Table 1), including nasal swabs of the caterer's nose, different foods, a swab from a kitchen sink at the site of the celebration, as well as the stool sample of one patient.

FT-IR was used for rapid species identification and for a preliminary determination of the degree of relatedness of the isolates. We compared the results of the FT-IR in a dendrogram (Fig. 1) that also included a number of *S. aureus* strains for reference that were not involved in the outbreak investigation (strains designated CVUAS, DSM, and ATCC). Seven of the eight obtained *S. aureus* isolates clustered together, including the isolate obtained from the stool sample of a patient, while SA_4 obtained from raw chicken represented the only isolate located on another branch of the similarity tree. This was confirmed by *spa* typing that assigned SA_4 to t002, while assigning the seven other *S. aureus* isolates to t018.

DNA microarray was used to further characterize the seven isolates assigned to *spa* type t018. All tested isolates exhibited either identical profiles or differed only in the result determined for one of over 250 probes. DNA microarray confirmed that all tested isolates represent *S. aureus* and assigned the isolates to clonal complex 30. Among others, genes encoding enterotoxins SEA/SEG/SEI (*entA, entA-320E, entG, entI*), the enterotoxin-like superantigens SEM/SEN/SEO/SEU (*entM, entN, entO, entU*), as well as TSST-1 (*tst-1*) were detected. For a full list of hybridization results of all isolates, see Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/fpd).

PFGE patterns are depicted in Figure 2. The seven isolates that were assigned to t018 and exhibited highly similar FT-IR and DNA microarray profiles also display identical PFGE patterns (SA_1, SA_2, SA_3, SA_5, SA_6, SA_7, SA_8).

Table 1. Characteristics of All *Staphylococcus aureus* Isolated by the Investigative Team

		RPLA toxin detection						
ID	*Source*	*SEA*	*SEB*	*SEC*	*SED*	*TSST-1*	*Spa type*	*CFU/g food*
SA_1	Nasal swab of caterer's nose #1	+	—	—	—	+	t018	n.a.
SA_2	Nasal swab of caterer's nose #2	+	—	—	—	+	t018	n.a.
SA_3	Sink at the site of the celebration	+	—	—	—	+	t018	n.a.
SA_4	Raw chicken filled with broccoli	—	—	—	—	—	t002	10^2
SA_5	Cooked meat	+	—	—	—	+	t018	10^6
SA_6	Meat-filled pancake	+	—	—	—	+	t018	10^6
SA_7	Watermelon	+	—	—	—	+	t018	10^4
SA_8	Stool sample of patient	+	—	—	—	+	t018	n.a.

n.a., not applicable.

The questionnaires revealed that the 2-year-old child had exclusively eaten pancakes filled with minced chicken and that this dish had also been ingested by all other patients. The average incubation time before onset of symptoms equalled 4.2 h. The clinical symptom common in all patients was emesis, while only some patients also reported diarrhea, abdominal pain/cramps, nausea, fever (39–40°C), cardiovascular problems, ague, and aching limbs. The 2-year-old child suffered from severe dehydration.

Discussion

FT-IR analysis as well as *spa* typing suggested that the seven *S. aureus* isolates SA_1-3 and SA_5-8 are very closely related. *Spa* typing assigned these strains to t018, a *spa* type previously found in common methicillin-resistant *S. aureus* clones in the United Kingdom and Denmark (Bartels *et al.*, 2009; Khandavilli *et al.*, 2009), as well as *S. aureus* strains associated with nasal colonization, infections, and food poisoning (Wattinger *et al.*, 2012). These seven highly similar isolates originated from food samples, nasal swabs of the caterer, as well as a stool sample from a patient.

In contrast, isolate SA_4 exhibited a different *spa* type and FT-IR spectrum and could not be linked to the outbreak.

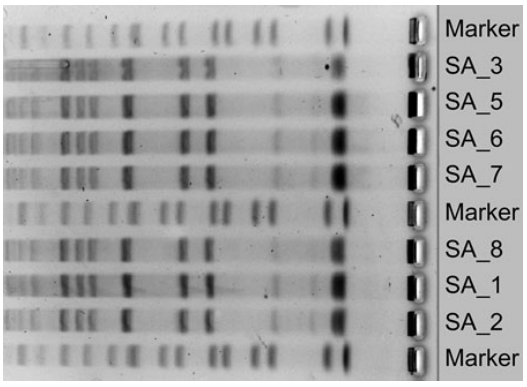

FIG. 2. The seven isolates that were assigned to t018 and exhibited highly similar Fourier transform infrared spectroscopy and DNA microarray profiles also display identical pulsed-field gel electrophoresis patterns (SA_1, SA_2, SA_3, SA_5, SA_6, SA_7, SA_8). Molecular size standards were used in lanes 1, 6, and 10. Lanes 2–5, as well as lanes 7–9, each contain a different staphylococcal isolate.

Therefore, only the seven isolates assigned to t018 were further characterized by DNA microarray and PFGE. The identical PFGE patterns and highly similar DNA microarray profiles of these isolates suggest that they represent the same *S. aureus* strain.

DNA microarray showed that the enterotoxin genes *sea, seg, sei* encoding SEA, SEG, and SEI were present, consistent with the identification of this strain as the source of the food-poisoning outbreak. Expression of the *sea* gene was confirmed by RPLA, detecting the SEA protein. SEA represents the most common enterotoxin recovered from food-poisoning outbreaks in the United States (77.8%) and was shown to exhibit exceptionally high emetic activity, with a total dose of only 200 ng being sufficient to cause gastroenteritis (Balaban *et al.*, 2000).

We detected isolates of the same SEA producing *S. aureus* strain in samples of the nose of the caterer, different foodstuffs served at the celebration including meat-filled pancakes, and the stool sample of one patient. It is therefore highly likely that the caterer inadvertently contaminated the food and that the organism was able to multiply and produce enterotoxins due to insufficient refrigeration before and/or during the buffet.

Conclusions

This is one of only a few studies (Argudín *et al.*, 2010; Johler *et al.*, 2010) that was able to link a staphylococcal food poisoning outbreak to its source. We were able to match an enterotoxigenic strain in the stool sample of a patient to isolates of the same strain obtained from food and the nasal cavity of a food handler. The strain produced SEA and TSST-1, and was also found to exhibit the genes encoding enterotoxins SEG and SEI, as well as the enterotoxin gene cluster *egc*.

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Disclosure Statement

No competing financial interests exist.

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6.2 Publication 14

Outbreak of staphylococcal food poisoning among children at a Swiss boarding school due to soft cheese made from raw milk

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Outbreak of staphylococcal food poisoning among children and staff at a Swiss boarding school due to soft cheese made from raw milk

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ABSTRACT

On October 1, 2014, children and staff members at a Swiss boarding school consumed Tomme, a soft cheese produced from raw cow milk. Within the following 7 h, all 14 persons who ingested the cheese fell ill, including 10 children and 4 staff members. Symptoms included abdominal pain and violent vomiting, followed by severe diarrhea and fever. We aim to present this food poisoning outbreak and characterize the causative agent. The duration of the incubation period was dependent of the age of the patient: 2.5 h in children under 10 yr of age, 3.5 h in older children and teenagers, and 7 h in adults. The soft cheese exhibited low levels of staphylococcal enterotoxin (SE) A (>6 ng of SEA/g of cheese) and high levels of staphylococcal enterotoxin D (>200 ng of SED/g of cheese). Counts of 10^7 cfu of coagulase-positive staphylococci per gram of cheese were detected, with 3 different *Staphylococcus aureus* strains being present at levels $>10^6$ cfu/g. The 3 strains were characterized using *spa* typing and a DNA microarray. An enterotoxin-producing strain exhibiting *sea* and *sed* was identified as the source of the outbreak. The strain was assigned to *spa* type t711 and clonal complex 8, and it exhibited genetic criteria consistent with the characteristics of a genotype B strain. This genotype comprises bovine *Staph. aureus* strains exclusively associated with very high within-herd prevalence of mastitis and has been described as a major contaminant in Swiss raw milk cheese. It is therefore highly likely that the raw milk used for Tomme production was heavily contaminated with *Staph. aureus* and that levels further increased due to growth of the organism and physical concentration effects during the cheese-making process. Only a few staphylococcal food poisoning outbreaks involving raw milk products have been described. Still, in view of this outbreak and the possible occurrence of other foodborne pathogens in bovine milk, consumption of raw milk and soft cheese produced from raw milk constitutes a health risk, particularly when young children or other members of sensitive populations are involved.
Key words: outbreak investigation, *Staphylococcus aureus*, raw milk cheese, genotype B

INTRODUCTION

Staphylococcal food poisoning (**SFP**) is one of the most prevalent causes of foodborne intoxication worldwide, resulting in an estimated 241,148 cases and 6 deaths in 2006 in the United States alone (Scallan et al., 2011). After a short incubation period of 2 to 6 h, patients exhibit nausea, followed by violent vomiting and diarrhea (Tranter, 1990). As clinical symptoms are typically self-limiting, it is estimated that only 10% of SFP patients visit a hospital (Holmberg and Blake, 1984). Consequences include pronounced underreporting of the disease and scarce scientific data on the characteristics of *Staph. aureus* strains causing SFP.

Staphylococcal food poisoning is caused by consumption of staphylococcal enterotoxins (**SE**) formed by *Staph. aureus* in food. To date, more than 20 different SE and SE-like superantigens have been described (Hennekinne et al., 2012), but only a few have been demonstrated to elicit an emetic response in a monkey feeding assay. Staphylococcal enterotoxins that have been shown to exhibit emetic activity include the classical enterotoxins SEA, SEB, SEC, SED, and SEE, and, to a limited degree, some newly described enterotoxins (Thomas et al., 2007).

In SFP outbreak investigations, identification of the causative strain can be challenging, as SE are highly heat resistant. Even when the organism is inactivated and can no longer be isolated from a food item, the highly stable enterotoxins formed by *Staph. aureus* can still cause SFP (Le Loir et al., 2003). In addition,

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identification of the causative strain in an outbreak investigation is aggravated by the high prevalence of *Staph. aureus* in humans and animals. *Staphylococcus aureus* persistently colonizes the anterior nares of 20 to 30% of the human population (van Belkum et al., 2009), causes a multitude of infections in humans and livestock, and can be isolated from a wide range of food items (Baumgartner et al., 2014). The organism also represents a common cause of bovine mastitis and can be detected in bulk tank milk at prevalence rates of 27 to 42% (Oliver et al., 2009).

On October 1, 2014, children and staff members at a Swiss boarding school consumed Tomme, a soft cheese produced from raw milk. Within 7 h, all 14 persons who had consumed the cheese fell ill, among them 10 children and 4 members of the staff. Based on the short incubation time, as well as the clinical symptoms, SFP due to consumption of the raw milk cheese was considered a possible cause of the outbreak.

MATERIALS AND METHODS

Enumeration and Isolation of Coagulase-Positive Staphylococci, Cell Lysis, and DNA Extraction

Coagulase-positive staphylococci (**CPS**) present in the Tomme soft cheese (that had been stored at 4°C) were enumerated according to EN ISO 6888-2 (ISO, 1999).

Different morphologies of colonies forming an opaque fibrin halo on rabbit plasma fibrinogen agar (Oxoid, Basel, Switzerland) after 48 h of incubation at 37°C were subcultured on 5% sheep blood agar (Oxoid) and incubated overnight at 37°C. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and following the manufacturer's instructions.

spa Typing

The polymorphic X region of the *spa* gene was determined as previously described (Wattinger et al., 2012). Briefly, *spa* was amplified using spa-1113f (5′-TAA AGA CGA TCC TTC GGT GAG C-3′) and spa-1514r primers (5′-CAG CAG TAG TGC CGT TTG CTT-3′) (Aires-de-Sousa et al., 2006) and the GoTaq PCR system (Promega AG, Dübendorf, Switzerland) at the following reaction conditions: (1) 5 min at 94°C; (2) 35 cycles of 45 s at 94°C, 45 s at 60°C, and 90s at 72°C; and (3) 10 min at 72°C. The PCR amplicons were purified using the MinElute PCR Purification Kit (Qiagen). Sequencing was outsourced (Microsynth, Balgach, Switzerland) and *spa* types were determined using the *spa*-server (http://spa.ridom.de/; Harmsen et al., 2003).

Microarray-Based Genotyping

The *Staph. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany) was used to further characterize the *Staph. aureus* strains. This analytical tool can be used to predict clonal complex (**CC**) assignment (Monecke et al., 2008) and to determine the presence or absence of over 300 resistance and virulence genes, including genes encoding the major SE (*sea*–*see*), newly described SE (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *seq*, *ser*), and enterotoxin-like superantigens (*selm*, *seln*, *selo*, *selu*).

Detection of SE in Cheese

To screen for all major SE, an extract of the cheese sample was prepared and analyzed using SET2 miniVIDAS (bioMérieux, Lyon, France) according to the manufacturer's recommendations. The SET-RPLA (staphylococcal enterotoxin–reversed passive latex agglutination) kit (Oxoid) was subsequently used to enable semiquantitative detection of SEA, SEB, SEC, and SED.

RESULTS AND DISCUSSION

All persons who consumed the raw milk cheese fell ill, among them 10 children and 4 members of the staff (Table 1). Although the average incubation time was 4.4 h, the individual duration of the incubation period depended on the age of the patient. Only 2.5 h after consumption of the cheese, the 2 youngest children (age 8 and 9 yr) complained about abdominal pain, fever, and aching limbs that progressed quickly to emesis, followed by severe diarrhea, and fever. One hour later, the older children (10–16 yr) exhibited the same symptoms, followed 3.5 h later by the adults (31–57 yr). One person sought medical care and was treated.

We detected 10^7 cfu presumptive CPS per gram of cheese in the Tomme sample. Different morphologies of coagulase-positive colonies exhibiting a phenotype consistent with *Staph. aureus* were visible on RPF agar (Figure 1), indicating contamination of the product with more than one *Staph. aureus* strain. Using SET2 miniVIDAS to screen for major SE, the cheese tested positive for SEA, SEB, SEC, SED, and SEE in 25 g of product. Subsequently, the SET-RPLA kit was used for semiquantitative detection of SEA, SEB, SEC, and SED, identifying low levels of SEA (>6 ng of SEA/g of cheese) and high levels of SED (>200 ng of SED/g of cheese) in the Tomme soft cheese. Staphylococcal enterotoxin A is the most common SE recovered from food-poisoning outbreaks (78%) and is reported to cause symptoms of intoxication in humans at a total dose of

Table 1. Overview of 10 children and 4 staff members who suffered from clinical signs of staphylococcal intoxication[1]

Patient	Sex	Age (yr)	Incubation time (h)	Symptoms
1	M	8	2.5	Abdominal pain, emesis, diarrhea, fever
2	M	9	2.5	Abdominal pain, emesis, diarrhea, fever
3	M	10	3.5	Abdominal pain, emesis, diarrhea, fever
4	M	13	3.5	Abdominal pain, emesis, diarrhea, fever
5	M	13	3.5	Abdominal pain, emesis, diarrhea, fever
6	M	14	3.5	Abdominal pain, emesis, diarrhea, fever
7	M	15	3.5	Abdominal pain, emesis, diarrhea, fever
8	F	15	3.5	Abdominal pain, emesis, diarrhea, fever
9	F	15	3.5	Abdominal pain, emesis, diarrhea, fever
10	M	16	3.5	Abdominal pain, emesis, diarrhea, fever
11	M	31	7	Abdominal pain, emesis, bloody diarrhea, fever
12	M	33	7	Abdominal pain, emesis, diarrhea, fever
13	M	41	7	Abdominal pain, emesis, diarrhea, fever
14	F	57	7	Abdominal pain, emesis, diarrhea, fever

[1]With an average incubation time of 4.4 h, the onset of symptoms was earlier in children than in adults.

only 200 ng of SEA (Balaban and Rasooly, 2000). In a monkey feeding assay, 25 μg of SEA/kg of BW induced emesis, and in the house musk shrew, the 50% emetic dose (ED_{50}) for peroral administration equaled 32 μg of SEA/kg of BW (Hu and Nakane, 2014). Although SED represents the second most common SE and can be detected in 38% of SFP outbreaks (Balaban and Rasooly, 2000), there is no comparable data on the effect of SED after oral intake. However, intraperitoneal injection of a total dose of 40 μg of SED was shown to have an emetic effect in the house musk shrew (Hu and Nakane, 2014).

Three different *Staph. aureus* strains (SA_1, SA_2, SA_3) were isolated from the cheese and were further characterized by *spa* typing (Wattinger et al., 2012; Table 2). All 3 strains were present in the cheese sample at levels higher than 10^6 cfu/g, with SA_1 occurring at the highest level. A DNA microarray analysis was used to identify the *Staph. aureus* isolate that produced the major SE previously detected and to generate a virulence and resistance gene profile of the strains. Major staphylococcal enterotoxin genes were only detected in SA_1, a strain that exhibited both *sea* and *sed* (Table 2). Strain SA_1 was assigned to CC8, a clonal complex frequently detected among strains isolated from humans, animals, and food products. Based on the enterotoxins SEA and SED detected by SET-RPLA and the enterotoxin genes *sea* and *sed* detected in the microarray, *Staph. aureus* strain SA_1 isolated from the Tomme raw milk cheese was identified as the source of the outbreak.

Strain SA_1 was assigned to *spa* type t711 and CC8. The *spa* type t711 is commonly detected among bovine mastitis isolates in Switzerland (Johler et al., 2011; Sakwinska et al., 2011) and has also been described in association with infections in humans, including methicillin-resistant *Staph. aureus* (MRSA) infections caused by the USA300 clone (Yabe et al., 2010). Although SA_1 exhibited genes involved in β-lactam resistance (*blaZ*, *blaI*, *blaR*), no genes conferring methicillin resistance were detected. Strain SA1 belongs to CC8, a clonal complex frequently linked to SFP outbreak strains and staphylococcal infections in humans and animals (Monecke et al., 2009; Wattinger et al., 2012; Resch et al., 2013). In Switzerland, 13 to 36% of the *Staph. aureus* isolated from bovine mastitis milk (Sakwinska et al., 2011; Moser et al., 2013) and 12% of the *Staph. aureus* isolated from ready-to-eat foods (Baumgartner et al., 2014) belong to CC8.

Strain SA_1 exhibits genetic criteria (*sea*, *sed*, *sej*, CC8) consistent with the characteristics of a genotype

Table 2. Characteristics of the 3 *Staphylococcus aureus* strains isolated from the Tomme soft cheese made of raw milk, including the presence of genes encoding staphylococcal enterotoxins (SE)

Strain ID	Major SE genes					Genes encoding newly described SE and SE-like superantigens	*spa* type	*agr* type	CC[1]
	sea	*seb*	*sec*	*sed*	*see*				
SA_1	+	−	−	+	−	*sej, ser*	t711	agrI	CC8
SA_2	−	−	−	−	−	*egc* cluster (*seg*, *sei*, *selm*, *seln*, *selo*, *selu*)	t018	agrII	CC705
SA_3	−	−	−	−	−	*egc* cluster (*seg*, *sei*, *selm*, *seln*, *selo*, *selu*)	t458	agrI	CC20

[1]Assignment to clonal complexes (CC) based on DNA microarray predictions.

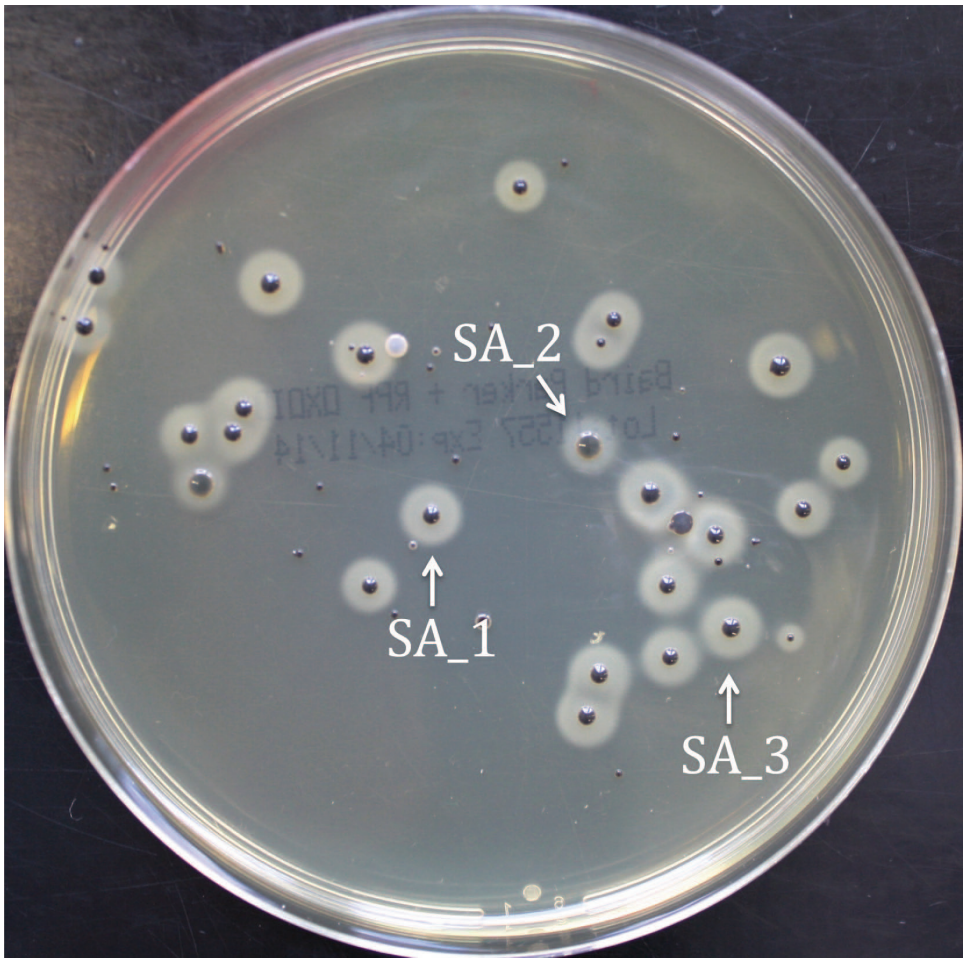

Figure 1. Rabbit plasma fibrinogen agar (RPF; Oxoid, Basingstoke, UK) used for enumeration of coagulase-positive staphylococci. Different morphologies of colonies consistent with a *Staphylococcus aureus* phenotype were visible on RPF (dilution 1:1,000,000), indicating contamination of the product with more than one *Staph. aureus* strain. The 3 phenotypes slightly differed in colony size, coloration, and the size of the fibrin reaction formed due to coagulase activity.

B strain (Boss et al., 2011; Moser et al., 2013). This genotype comprises bovine *Staph. aureus* strains exclusively associated with very high (up to 65%) within-herd prevalence of mastitis (Graber et al., 2009) and has been described as a major contaminant in Swiss raw milk cheese (Hummerjohann et al., 2014). It is therefore highly likely that the raw milk used for Tomme production was strongly contaminated with *Staph. aureus*. Only a few SFP outbreaks involving raw milk products have been described and many are associated with goat or sheep milk products rather than products made of bovine raw milk (De Buyser et al., 2001; Giezendanner et al., 2009; Ostyn et al., 2010). During the production process of soft cheese made from raw milk, *Staph. aureus* levels can further increase due to both growth of the organism, as well as physical concentration effects that result in an estimated increase of 1 $\log_{10}$ (Peng et al., 2013).

CONCLUSIONS

In view of this outbreak and the possible occurrence of other foodborne pathogens such as Shiga toxin-producing *Escherichia coli* in bovine milk, consumption of raw milk and soft cheese produced from raw milk constitutes a health risk, in particular when young children or members of other sensitive populations are involved.

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6.3 Publication 15

Further evidence for staphylococcal food poisoning outbreaks caused by *egc*-encoded enterotoxins

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Article

Further Evidence for Staphylococcal Food Poisoning Outbreaks Caused by *egc*-Encoded Enterotoxins

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Abstract: Staphylococcal food poisoning represents the most prevalent foodborne intoxication worldwide. It is caused by oral intake of enterotoxins preformed by *Staphylococcus aureus* in food. The relevance of newly described enterotoxins in outbreaks of staphylococcal food poisoning is controversially discussed. Although the staphylococcal enterotoxins SEG, SEI, SEM, SEN, and SEO elicit emesis in a monkey feeding assay, there has been no conclusive proof of their emetic activity in humans. In this study, we provide further evidence suggesting that one of these enterotoxins or a combination of SEG, SEI, SEM, SEN, and SEO cause staphylococcal food poisoning. We investigated two outbreaks registered with the Swiss Federal Office of Public Health, in which only *Staphylococcus aureus* strains harboring the *egc* cluster, including *seg*, *sei*, *sem*, *sen*, and *seo* linked to typical signs of staphylococcal food poisoning were isolated. The outbreaks were caused by consumption of raw goat cheese and semi-hard goat cheese, and were linked to strains assigned to CC45 (*agr* type I) and CC9 (*agr* type II), respectively. These outbreaks provide further evidence that newly-described staphylococcal enterotoxins are likely to cause staphylococcal food poisoning in humans.

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Keywords: staphylococcal food poisoning; enterotoxin; *seg*; *sei*; outbreak investigation; *Staphylococcus aureus*

1. Introduction

The CDC (Centers for Disease Control) estimates that 240,000 cases of Staphylococcal Food Poisoning (SFP) occur each year in the US, leading to hopitalization in 1000 cases and to six deaths [1]. In the EU, the number of SFP outbreaks is rising, with 386 SFP outbreaks reported in 2013 [2]. The causative agents are staphylococcal enterotoxins (SEs) preformed by *Staphylococcus (S.) aureus* in food. Within two to six hours after ingestion of food containing SEs, patients present with symptoms of acute gastroenteritis, including violent vomiting and diarrhea [3]. As SFP is typically self-limiting, with only 10% of patients [4] seeking medical care, there are only a few SFP outbreaks, in which a causative *S. aureus* strain and its respective enterotoxins were unambiguously identified.

A variety of SEs and SE-like superantigens has been described. All classical SEs (SEA-SEE) and, to a lesser degree, also most of the newly described SEs, including SEG and SEI, can elicit an emetic response in a monkey feeding assay [5,6]. However, it was shown that strains harboring *seg* and *sei* only produce very low levels of SEG and SEI [7] and only weak emetic activity was demonstrated for SEI [8]. Thus, only for the classical SEs [9] and SEH [10–12], there is evidence demonstrating emetic activity in humans. Still, in recent years, there have been a growing number of studies indicating that SEG and SEI may be responsible for cases of SFP in humans [13,14]. In the context of large studies characterizing a multitude of *S. aureus* strains from various outbreaks, one outbreak caused by an isolate harboring *seg*, and two outbreaks caused by isolates harboring *seg* and *sei* have been mentioned [13–15]. However, as no information on the outbreak investigations was provided, it is impossible to evaluate whether sufficient evidence was present to unambiguously link the strains as the cause of the SFP cases.

The discussion is further fueled by the fact that in many outbreaks strains exhibiting both classical and newly described enterotoxin genes can be detected, but only classical enterotoxins can be identified in food and feces by commercially available immunological based methods. Thus, if food and feces samples yield positive results for even very small quantities of classical enterotoxins, outbreaks will be reported to be due to these enterotoxins, even if *seg*, *sei*, *sem*, *sen*, or *seo* genes are present. If, however, food and feces samples yield negative results for classical enterotoxins, but strains harboring *seg*, *sei*, *sem*, *sen*, or *seo* were found, the outbreak may not be reported at all as investigators question the emetic activity of SEG, SEI, SEM, SEN, and SEO in humans.

In a recent study investigating clonality and genetic characteristics of *S. aureus* strains isolated from ready-to-eat foods in Switzerland [16], we identified two cases of SFP, in which only strains exhibiting the newly-described SE genes *seg*, *sei*, *sem*, *sen*, and *seo* had been detected. In addition, in September 2014, yet another SFP outbreak linked to a *S. aureus* strain exhibiting *seg*, *sei*, *sem*, *sen*, and *seo* occurred in Switzerland. In this study, we present collected evidence from two outbreak investigations, suggesting that newly-described enterotoxins can indeed cause SFP in humans.

2. Results

2.1. Outbreak 2007

On 4 June 2007, two people purchased Robiola type fresh cheese made from thermized goat milk at a store in Ticino, the Italian-speaking part of Switzerland. While one of them stored the cheese at refrigeration temperatures to serve it to his family at dinner, the other gave it as a present to a friend who consumed the cheese right away. Within 1.5–3 h after consumption, all five people that had ingested the cheese exhibited signs of acute gastroenteritis, such as nausea, abdominal cramps, vomiting, and, in some cases, diarrhea (Table 1).

Table 1. Outbreak due to Robiola goat cheese on 4 June 2007.

Patient	Age	Sex	Hospitalized	Clinical signs	Incubation time
Patient 1	54	M	Yes	Nausea, vomitus, abdominal cramps	1.5–2 h
Patient 2	54	F	Yes	Nausea, vomitus, abdominal cramps, diarrhea	1.5–2 h
Patient 3	23	M	Yes	Nausea, vomitus, abdominal cramps, diarrhea	1.5–2 h
Patient 4	21	M	Yes	Nausea, vomitus, abdominal cramps	1.5–2 h
Patient 5	38	M	No	Nausea, vomitus, abdominal cramps, diarrhea	2.5–3 h

Thirteen Robiola samples were taken from households of the patients, as well as from Robiola cheese produced by the manufacturer on the day after the outbreak. While the maximum level of coagulase positive *Staphylococci* (CPS) detected among samples obtained from the manufacturer was 4.6×10^5 CfU/g cheese, samples obtained from cheese associated with illness exhibited between 6.7×10^6 and 2.6×10^7 CfU/g cheese. As all cheese samples tested negative for the classical enterotoxins SEA-SEE using Vidas SET2, three *S. aureus* strains were selected for further characterization by DNA microarray (Table 2) to test not only for genes encoding the classical enterotoxins (*sea-see*), but also for genes encoding the newly described enterotoxins (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, *selu*). The only enterotoxin genes detected by DNA microarray were the genes of the enterotoxin gene cluster *seg*, *sei*, *sem*, *sen*, and *seo*. Microarray profiles showed that all three isolates represented the same *S. aureus* strain and belong to clonal complex (CC) 45 and *agr* type I. Complete microarray hybridization results for all three outbreaks are provided as a supplemental file. No *ses* and *set* enterotoxin genes were detected by PCR screening.

Table 2. Selected Robiola goat cheese samples obtained during the 2007 outbreak from households of SFP patients. *S. aureus* isolates were characterized by DNA microarray to test for genes encoding the classical, as well as newly described enterotoxins.

ID	CPS in CfU/g	Source	SET Vidas	Classical SE genes	Newly described SE genes	Clonal complex
SFP1_1	7.1×10^6	Patients 1–4	No SEA-SEE	None	*seg*, *sei*, *sem*, *sen*, *seo*	CC45
SFP1_2	7.6×10^6	Patients 1–4	No SEA-SEE	None	*seg*, *sei*, *sem*, *sen*, *seo*	CC45
SFP1_3	2.6×10^7	Patient 5	No SEA-SEE	None	*seg*, *sei*, *sem*, *sen*, *seo*	CC45

2.2. Outbreak 2014

On 7 September 2014, an SFP outbreak due to Formagella d'alpe goat cheese occurred in the Ticino region, the Italian-speaking part of Switzerland. Five people consumed the semi-hard goat cheese made

from raw milk, and fell ill within the following 6 h (average incubation time 3.8 h), exhibiting acute symptoms of gastroenteritis, such as nausea and vomiting (Table 3).

Table 3. Outbreak due to consumption of a semi-hard cheese from raw goat milk (Formagella d'alpe) on 7 September 2014.

ID	Age	Sex	SFP	Incubation time	Clinical signs	Consumed food items
Person 1	80	F	Yes	6 h	Nausea, vomitus	Semi-hard cheese from raw goat milk, fresh cheese, potatoes
Person 2	50	M	Yes	3.5 h	Nausea, vomitus	Semi-hard cheese from raw goat milk, fresh cheese, potatoes
Person 3	52	F	Yes	4.5 h	Nausea, vomitus	Semi-hard cheese from raw goat milk, fresh cheese, potatoes
Person 4	48	F	Yes	2.5 h	Nausea, vomitus	Semi-hard cheese from raw goat milk
Person 5	14	M	Yes	2.5 h	Nausea, vomitus	Semi-hard cheese from raw goat milk
Person 6	42	M	No	-	None	Semi-hard cheese from raw goat milk

The investigative team performed a case control study, in which the semi-hard goat cheese was identified as the cause of the outbreak (odds ratio = 25). The cheese was a Formagella d'alpe that had been produced on 1 August and had, thus, been ripened for five weeks before consumption. The cheese tested negative for the classical enterotoxins SEA-SEE in the mini Vidas SET2. A total of 2.6×10^3 CfU CPS per g cheese were detected. Single colonies consistent with a *S. aureus* phenotype on rabbit plasma fibrinogen agar were screened for genes encoding enterotoxins. Four different *S. aureus* strains were detected (Table 4). Three of these strains exhibited no enterotoxin genes. Only one strain (SFP3_1) exhibited the genes of the enterotoxin gene cluster *seg*, *sei*, *sem*, *sen*, and *seo* but no other enterotoxin genes including *ses* and *set*. SFP3_1 was assigned to CC9 and *agr* type II.

Table 4. *S. aureus* strains isolated from the semi-hard goat cheese associated with the 2014 outbreak of SFP in Ticino. Strains were characterized by DNA microarray to test for genes encoding the classical, as well as newly described enterotoxins. No classical enterotoxins (SEA-SEE) were detected in the cheese samples by mini Vidas SET2.

ID	Clonal complex	Classical SE genes	SEA-SEE (mini Vidas SET2)	Newly described SE genes
SFP2_1	CC9	None	None	*seg*, *sei*, *sem*, *sen*, *seo*
SFP2_2	CC45	None	None	None
SFP2_3	CC130	None	None	None
SFP2_4	CC522	None	None	None

3. Discussion

The results of our study illustrate that risk factor analysis in SFP outbreak investigations should not be solely based on CPS counts. In the outbreak in 2007 due to Robiola fresh cheese from thermized goat milk, CPS count per g food considerably exceeded the legal limit of 10^5 CfU/g food, which is considered a health risk (EU 2073:2005). However, in the outbreak in 2014, we detected only 2.6×10^3 CfU of CPS per g cheese at the time of consumption. However, by this time, the semi-hard cheese from raw goat

milk had been ripened for five weeks. In semi-hard raw milk cheese ripened at 10 °C and 92% air humidity, CPS counts were shown to peak around day seven of the ripening process and then gradually decline until they commonly fall below the detection limit at week nine of the ripening process [17]. The strains causing the SFP outbreaks in this study were assigned to CC9 and CC45. CC9 is particularly frequently found in strains isolated from caprine mastitis milk [18].

In this study, we present outbreaks, in which only strains harboring *seg* and *sei*, and other genes encoded by *egc* were detected. We omitted any SFP outbreaks, in which classical enterotoxin genes or *seh* were detected in the same or another *S. aureus* strain.

In both outbreaks described in this study, the causative strain carried *seg*, *sei*, *sem*, *sen*, *seo*, and *selu*. Currently, no data on emetic activity of SEIU is available. Administering SEM, SEN, and SEO to cynomolgus monkeys at 100 μg/kg showed weak emetic activity and resulted in vomiting of one in seven, two in six, and one in eight animals, respectively [6]. ED_{50} of classical enterotoxins in monkeys ranges between 0.9 and 20 μg/kg [19,20]. In rhesus monkeys, SEG and SEI provoke diarrhea or pronounced lethargy in all tested animals and emesis in four out of six (80 μg/kg SEG) and one out of four (150 μg/kg SEI) animals [8]. While the emetic activity of SEI in the rhesus monkey seems rather low, it provokes vomiting in the house musk shrew at an emetic activity comparable to SEA and far exceeding SEB, SEC, and SED [21].

Taking into consideration data from feeding assays, as well as reports of strains carrying *seg* and *sei* in association with intoxications [13–15], it is most likely that the SFP outbreaks described in this study were caused by SEG and/ or SEI. It is also highly likely that the semi-hard goat cheese that caused the SFP outbreak exhibited far higher CPS counts earlier in the ripening process and that during this phase large amounts of enterotoxins were produced by *S. aureus*. Even when the number of viable cells of the organism is reduced, the extremely stable enterotoxins remain emetically active and can cause SFP [22].

Interestingly, both presumptive SEG and SEI associated SFP outbreaks described in this study are linked to goat milk products. This is consistent with previous studies reporting that the enterotoxin gene combinations *sec/seg/sei* and *seg*/*sei* were frequently detected among *S. aureus* strains isolated from cheese made of raw goats' milk [23]. Interestingly, both SFP outbreaks were linked to goat milk products. As there are currently no suitable methods for detection of SEG and SEI or other newly-described enterotoxins in food and feces, further research will be necessary to determine the individual role of these enterotoxins in SFP outbreaks.

4. Materials and Methods

4.1. Screening for Staphylococcal Enterotoxins

Vidas SET and mini Vidas SET2 (bioMérieux, Marcy d'Etoile, France) were used to screen for the classical enterotoxins SEA-SEE in food. Cheese extracts were prepared and processed following the manufacturer's instructions.

4.2. Bacterial Isolation and Identification

Columbia agar with 5% sheep blood (bioMérieux, Marcy d'Etoile, France) and Baird Parker Rabbit Plasma Fibrinogen (RPF, Oxoid, Basel, Switzerland) agar were used to screen for *S. aureus*. The number

of coagulase-positive *Staphylococci* per g food was determined using the plate count technique on RPF and following the EN ISO 6888-2 protocol. Several presumptive *S. aureus* colonies were selected from RPF agar and were further characterized.

4.3. Microarray-Based Genotyping

The *S. aureus* Genotyping Kit 2.0 ArrayStrip platform (Alere Technologies GmbH, Jena, Germany) was used for DNA microarray profiling according to the manufacturer's instructions. DNA microarray determines the presence/absence of over 300 allelic variants of resistance and virulence genes including genes encoding classical (*sea-see*), and newly-described enterotoxins and enterotoxin-like superantigens (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, *selu*) [24]. It also allows for *S. aureus* species confirmation and predicts assignment of the tested isolates to clonal complexes, as well as *agr* types [24]. The microarray has been used to detect enterotoxin genes in *S. aureus* linked to outbreaks [25,26] or isolated from different foods [16,27,28].

4.4. PCR Screening for ses and set

As the DNA microarray does not include probes targeting *ses* and *set*, screening for these enterotoxin genes was performed using a PCR approach, a GoTqy PCR system (Promega AG, Dübendorf, Switzerland) and primers are published elsewhere [29].

4.5. Odds Ratios

In the outbreak in Ticino in 2014, family members, as well as persons that had consumed semi-hard cheese from a different batch from the same producer were used as controls ($n = 6$). Information on food ingested up to 48 h before the outbreak was collected from all cases and controls. As all SFP patients had ingested the suspicious cheese, zero cases reported no exposure to the cheese ($c = 0$), and c was replaced by 1 to allow for OR calculation.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/2072-6651/7/3/0997/s1.

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Author Contributions

Sophia Johler and Roger Stephan conceived and designed the study. Petra Giannini, Marco Jermini, Jörg Hummerjohann, and Sophia Johler performed the experiments and analyzed the data. Andreas Baumgartner contributed strains and data. Sophia Johler wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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6.4 Publication 16

Investigation of a staphylococcal food poisoning outbreak combining case-control, traditional typing and whole genome sequencing methods, Luxembourg, June 2014

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Surveillance and outbreak report

Investigation of a staphylococcal food poisoning outbreak combining case–control, traditional typing and whole genome sequencing methods, Luxembourg, June 2014

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In June 2014, a staphylococcal food poisoning outbreak occurred at an international equine sports event in Luxembourg requiring the hospitalisation of 31 persons. We conducted a microbiological investigation of patients and buffet items, a case–control study and a carriage study of catering staff. Isolates of *Staphylococcus aureus* from patients, food and catering staff were characterised and compared using traditional typing methods and whole genome sequencing. Genotypically identical strains (sequence type ST8, *spa*-type t024, MLVA-type 4698, enterotoxin A FRI100) were isolated in 10 patients, shiitake mushrooms, cured ham, and in three members of staff. The case–control study strongly suggested pasta salad with pesto as the vehicle of infection (p<0.001), but this food item could not be tested, because there were no leftovers. Additional enterotoxigenic strains genetically unrelated to the outbreak strain were found in four members of staff. Non-enterotoxigenic strains with livestock-associated sequence type ST398 were isolated from three food items and two members of staff. The main cause of the outbreak is likely to have been not maintaining the cold chain after food preparation. Whole genome sequencing resulted in phylogenetic clustering which concurred with traditional typing while simultaneously characterising virulence and resistance traits.

Introduction

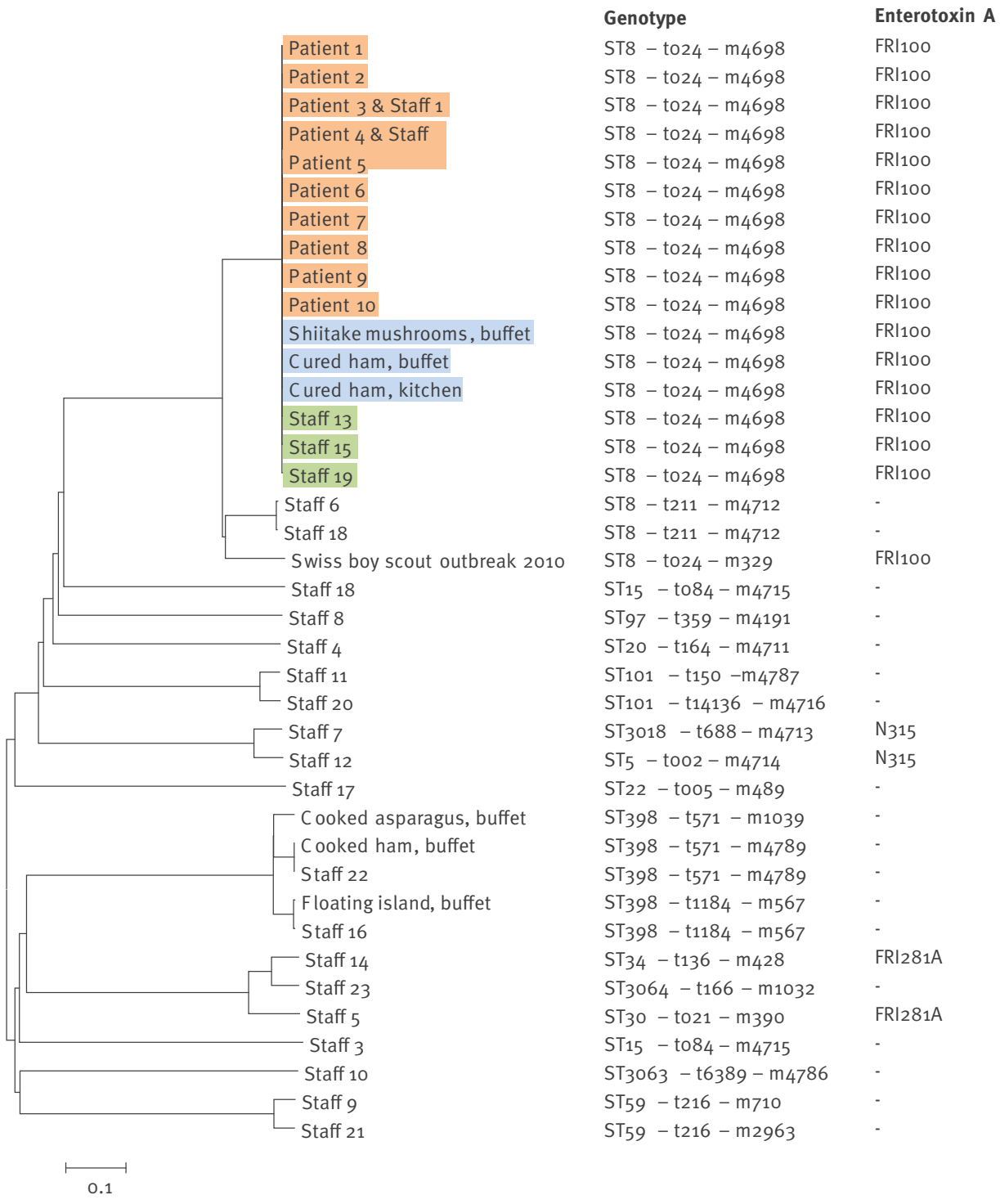
Food poisoning caused by enterotoxigenic *Staphylococcus aureus* is one of the most common foodborne diseases [1]. In France, which has a long-established foodborne disease surveillance system able to detect fairly rare events [2], staphylococcal food poisoning (SFP) has ranked in recent years as the first cause of foodborne outbreaks: of 1,288 reported foodborne outbreaks in 2012, 300 (23%) were due to SFP [3]. SFPs are thought to be under-reported for several reasons. First, because of the short duration of symptoms, only an estimated 10% of SFP patients visit a hospital [4]. Even if patients seek medical care, the physician often does not deem a stool analysis necessary. If a stool analysis is performed, the microbiological routine procedures often do not include testing for the presence of enterotoxigenic *S. aureus* unless specifically requested by the physician [5]. In addition, staphylococcal enterotoxin (SE) is highly stable and heat-resistant. Although the bacteria may have been inactivated by heating the food prior to consumption and can therefore be isolated neither from food nor the stool of the patient, the highly stable enterotoxins performed by *S. aureus* in the food may still be emetically active [6].

In contrast to most other gastrointestinal infections, the onset of SFP symptoms is very rapid, usually within a few hours after ingestion of the contaminated food. The median incubation period of aetiologically confirmed SFP outbreaks occurring in the United States between 1998 and 2008 was estimated to be four hours (5–95 percentile: two to seven hours) [7]. Symptoms in cases in these outbreaks typically included abdominal cramps (72%), vomiting (87%), and diarrhoea (89%). Fever (9%) was infrequently reported. The median duration of illness was 15 hours (5–95 percentile: 4–60 hours) [7].

Here, we report a SFP outbreak that occurred in a buffet restaurant at an international show-jumping event in Luxembourg in June 2014. A total of 31 persons had to be transferred by ambulance from the event site to emergency departments of three local hospitals. We describe findings of the ensuing epidemiological case–control study, the microbiological contamination

Figure

Clonal relationship between patient, food, and catering staff isolates, staphylococcal food poisoning outbreak, Luxembourg, 12–13 June 2014



A phylogenetic dendrogram (neighbour joining tree) was generated for 39 *Staphylococcus aureus* isolates based on the allelic profiles of 1,625 available of 1,878 queried MLST+ target genes. The scale bars indicate the number of differing alleles comprising the calculated distance. The colours represent the origin of outbreak-related strains (orange: stool samples from hospitalised patients; blue: food samples; green: throat or nose samples from colonised staff members). The genotype column shows the combined data of multilocus sequence typing (prefix ST), *spa*-typing (prefix t), and MLVA typing (prefix m).

Table

Results from analytical case–control study of food exposures, staphylococcal food poisoning outbreak, Luxembourg, June 2014

Food item at VIP buffet	Cases N (%)	Controls N (%)	Odds ratio	p value
Cooked ham	10 (45%)	6 (27%)	2.08 (0.50–9.04)	0.25
Cured ham	13 (59%)	10 (45%)	1.59 (0.41–6.28)	0.45
Grilled shrimps	12 (55%)	8 (36%)	1.95 (0.49–7.84)	0.28
Lamb	13 (59%)	11 (50%)	1.31 (0.33–5.18)	0.66
Melon	13 (59%)	12 (55%)	1.08 (0.27–4.30)	0.90
Panna cotta	13 (59%)	9 (41%)	1.93 (0.49–7.68)	0.29
Pesto pasta salad	18 (82%)	3 (14%)	27 (4.35–195.43)	<0.0001
Potato gratin	15 (68%)	13 (59%)	1.32 (0.31–5.59)	0.67
Potato salad	10 (45%)	11 (50%)	0.76 (0.19–2.94)	0.65
Prawns	3 (14%)	1 (5%)	3.16 (0.22–173.90)	0.32
Raw vegetables	13 (59%)	11 (50%)	1.31 (0.33–5.18)	0.66
Risotto	12 (55%)	9 (41%)	1.6 (0.41–6.31)	0.44
Ruccola salad	10 (45%)	10 (45%)	0.92 (0.24–3.57)	0.89
Salmon tartare	3 (14%)	2 (9%)	1.5 (0.15–19.7)	0.67

of food samples, and colonisation by *S. aureus* of catering employees at the event. In particular, we characterised the *S. aureus* isolates from patients, food items obtained from the buffet, and food handlers using traditional typing methods (PCR, *spa*-typing, and multilocus variable-number tandem repeat analysis (MLVA)), as well as whole genome sequencing.

The event

From 12 to 15 June 2014, an equestrian show-jumping event with approximately 140 participating international athletes and 300 horses took place in Luxembourg. Approximately one to three hours after eating a buffet lunch in the tented VIP restaurant on 12 June, 11 persons with symptoms of vomiting, diarrhoea, and prostration were taken by ambulance to the emergency departments of two hospitals where they received parenteral fluids. The official health inspection service was informed immediately of the incident and microbiological analysis of stool samples from hospitalised patients was ordered. Official food safety inspectors proceeded immediately to take samples from remaining buffet items for microbiological analysis. An inspection of the professional caterer's onsite restaurant and offsite kitchen did not reveal any major food safety deficiencies as specified in regulation (EC) 852/2004. The next morning, on 13 June, local newspapers announced salmon tartare as a potential culprit. A few hours after having the buffet lunch in the VIP restaurant on 13 June, a further 20 persons fell ill with the same symptoms and were transferred by ambulance to hospital emergency departments. The event organiser stopped serving any prepared meals for the remainder of the event. On 14 and 15 June, there were no further reports of gastrointestinal illness related to the event. While approximately 150-200 persons were estimated to have consumed the buffet lunch in the VIP restaurant on both days and a total of 31 persons were admitted to hospital emergency departments over the two days, the exact number of affected persons is unknown. There were no reports of illness among those people who ate at the other food-serving premises at the event: a non-VIP lunch buffet operated by the same caterer but with different menus, and a barbecue stall hosted by non-professional club members.

Methods

Microbiological examination of stool samples

Culture of stool samples for bacterial pathogens (including *Salmonella, Campylobacter* and verotoxigenic *Escherichia coli*) conducted in three hospital laboratories revealed the presence of *S. aureus* in ten patients and *Enterococcus* in one patient. Isolates of *S. aureus* were immediately referred to the National Health Laboratory for further molecular characterisation.

Case-control study

Following their recovery from illness and after the food samples had been analysed, eight cases who had been admitted to emergency care were contacted by telephone to get initial information on potential food exposures. All food items and symptoms reported by cases were included in a final questionnaire administered by telephone to 22 cases and 21 controls. Cases were defined as persons with sudden gastrointestinal illness (at least one symptom: vomiting, diarrhoea, abdominal cramps or nausea) who had eaten buffet lunch at the VIP restaurant on 12 or 13 June. Controls were defined as persons who had eaten buffet lunch at the VIP restaurant on 12 or 13 June, without any gastrointestinal symptoms. Non-hospitalised cases and controls were contacted using information provided by the event organiser.

Testing of food samples

Food samples were tested by accredited methods for aerobic plate count, *E. coli*, coagulase-positive staphylococci (ISO 6888-2:1999), *Salmonella,* and *Bacillus cereus*. The salmon tartare and floating island dessert samples were additionally tested for *Listeria monocytogenes*.

Staphylococcal carriage study among caterer's employees

Following the detection of *S. aureus* in patients, a staphylococcal carriage study was conducted on 19 and 20 June among the caterer's employees who worked in the onsite restaurant or in the offsite kitchen where buffet items were prepared, including the slicing of ham. Catering employees screened included waiters, cooks, and other kitchen staff. Throat and nose swabs were taken by doctors and sent the same day to the laboratory where they were streaked onto selective Chapman media (reference 51053, BioMérieux, Marcy l'Etoile, France).

Characterisation of S. aureus isolates and whole genome sequencing

Isolates of *S. aureus* obtained from patients, food, and catering employees were confirmed by MALDI-TOF mass spectrometry (Bruker, Brussels, Belgium). Confirmed isolates of *S. aureus* were further characterised for the presence of *nuc, mecA*, toxic shock syndrome toxin 1 (TSST-1), and Panton-Valentine leukocidine (PVL) [8] as well as genes coding for staphylococcal enterotoxins A (*sea*), B, C, D E, H, I, and J [9]. Isolates exhibiting *sea* were further characterised by sequencing the PCR products and compared to strains containing allelic *sea* variants FRI100, FRI287A, and N315. In addition, isolates were subjected to *spa*-typing [8] and MLVA typing [10]. Whole genome sequencing of isolates was performed on a Miseq Desktop Sequencer using the Nextera DNA sample preparation kit (Illumina, Eindhoven, The Netherlands) with an average coverage of 59 fold (range 27-140 fold). Antimicrobial resistance genes, virulence factors and multi-locus sequence types (MLST) were determined by submitting the raw read files to public webserver tools hosted by the Center for Genomic Epidemiology in Denmark [11-13]. After sequencing, whole genome MLST+ was conducted using the Seqsphere+ v2.3 pipeline (Ridom, Münster, Germany). Briefly, after trimming reads until the average quality was 30 in a window of 30 bases, the trimmed reads were mapped to the reference genome NC_002951.2 and the allelic profiles of 1,878 target genes were determined based on the MLST+ scheme developed previously [14]. Final phylogenetic analysis was based on 10 patient isolates, 6 food isolates, 22 carrier isolates of members of staff, and an isolate with *spa*-type t024 from an unrelated Swiss outbreak in 2010. The whole genome reads were deposited at the European Nucleotide Archive (study accession no. PRJEB7847). For the purpose of this study, we use the terminology 'genotype' to denote the combined typing results of MLST, *spa*- and MLVA typing.

Results

Fifteen (48%) of the 31 patients admitted by ambulance to emergency care were women. The median age was 35 years (range 14–58 years). Three patients were catering staff at the event. Six patients were non-residents of Luxembourg. Information on clinical symptoms was available for 22 of the patients and included diarrhoea (20 patients), nausea (19 patients), cramps (19 patients) and vomiting (17 patients). All admitted patients were discharged within 48 hours. All 10 isolates obtained from patients' stool samples represented the same genotype (MLST sequence type (ST)-8, *spa*-type t024, MLVA-type 4698), possessed genes encoding *sea* allele FRI100 and conferring penicillin resistance mediated by *blaZ*.

Results from the analytical epidemiological case–control study (Table) implicated consumption of pasta salad with pesto as the most likely vehicle of SFP. Eighteen of 22 cases reported eating this food item compared to 3 of 21 controls ($p<0.0001$). All 14 interviewed cases who had been hospitalised reported eating the pasta salad with pesto. Unfortunately, there were no leftovers of the pasta salad with pesto when sampling was taking place and so this dish was not available for microbiological testing. Eating cured ham or salmon tartare were not statistically significant risk factors ($p=0.45$). One interviewed patient reported not having eaten ham at the buffet for religious reasons.

Food samples

Isolates of *S. aureus* with a genotype identical to patient isolates (MLST ST-8, *spa*-type t024, MLVA-type 4698) were detected in cured ham samples (range <40–5,200 colony-forming units (CFU)/g and shiitake mushrooms (<40 CFU/g) sampled at the event site and in cured ham samples (enumeration range <40–120 CFU/g) obtained at the offsite catering kitchen where the ham was sliced and stored (Figure). Non-enterotoxigenic isolates of *S. aureus* with a different genotype to patient isolates were found in cooked asparagus (<40 CFU/g, MLST ST-398, *spa*-type t571, MLVA type 1039), the floating island dessert (<40 CFU/g, MLST ST-398, *spa*-type t1184, MLVA-type 567) and several samples of cooked ham (range 50–320 CFU/g, MLST ST-398, *spa*-type t571, MLVA-type 4789). Unsliced complete legs of cured and cooked hams obtained from the supplying butcher were negative for *S. aureus*. All 18 food items sampled from the event buffet were negative for *Salmonella* and *E. coli*. One food item (cooked asparagus) was positive for presumptive *Bacillus cereus* (840 CFU/g).

The pasta salad with pesto could not be sampled during food inspection, as there were no leftovers from this dish. The primary ingredients used to make the pesto sauce for the pasta salad (fresh basil, hard cheese, and pine nuts) were all negative for *S. aureus*.

Staphylococcal carriage study

Thirty-eight of the 49 catering employees at the event were screened for nasal/throat carriage of *S. aureus*.

Median age of the screened employees was 32.5 years (range 17–50 years), and 11 were women. Twenty-two employees were found to be colonised by *S. aureus:* three staff members were colonised by strains identical to those found in patients (Figure). Another four employees were colonised by *S. aureus* isolates exhibiting *sea,* but a different genotype than the outbreak strain. None of the seven employees colonised by isolates exhibiting *sea* reported wounds or gastrointestinal disease prior to the event. Overall, 17 different genotypes were observed among the 22 colonised employees. None of the isolates in food, patients, or catering employees were meticillin-resistant or exhibited *pvl*.

Whole genome sequencing

The whole genome phylogeny (Figure), as determined by 1,625 of 1,878 MLST and MLST+ target genes that were present in all 39 isolates, clearly delineated the outbreak isolates. *S. aureus* isolates found in 10 patients were identical to those isolated from cured ham, shiitake mushrooms and from three catering employees. Interestingly, the Luxembourg outbreak strain had 347 allele differences with a strain that led to the intoxication of 27 boy scouts in Switzerland in 2010, although both strains share a common *spa*-type t024 [15]. Two of the three food isolates which differed from the outbreak strain were also observed among catering employees. These belonged to livestock-associated sequence type ST398 with *spa*-types t571 or t1184.

Discussion

Studies of foodborne outbreaks, in which enterotoxigenic isolates were detected in patients, food, and food handlers, are rare [16-18]. Our report shows that, even in the era of whole genome sequencing, public health investigations of foodborne outbreaks remain very dependent on classical case–control investigations for interpretation of events. Whereas initial microbiological typing results suggested cured ham as the main vehicle for the intoxication, the case–control study clearly identified the pasta salad with pesto as the most likely source, which was no longer available for microbiological testing.

In our outbreak, there was good evidence that the pathogen responsible for the outbreak *was S. aureus*, because identical enterotoxigenic strains of *S. aureus* with a common *spa*-type but rare MLVA type were recovered from the stools of 10 hospitalised cases. Because three catering employees were colonised by a strain with the same genotype, it is likely that at least one of them may represent the source of food contamination, either via manual contact or through respiratory secretions [19]. However, because catering employees were screened a week after the outbreak, it cannot also be ruled out that some staff members became colonised only during or after the event [20].

One of the probable factors contributing to the outbreak may have been the unusually hot weather for the season, with maximum temperatures ranging between 25 °C and 32 °C during the week preceding the event, compared with a historical average of 21 °C. The food safety inspection at the catering facility revealed that a fridge had stopped working properly a few days prior to the event, although the catering staff denied using this fridge to store any of the dishes. The pasta salad with pesto was reported to have been pre-cooked and sealed into plastic bags in 2 kg portions, and then cooled down in a fast refrigeration unit. Nevertheless, the fact that *S. aureus* was detected in several dishes including cured and cooked ham, at concentrations up to 5,200 CFU/g, suggests that the cold chain before or during the event was interrupted to allow sufficient microbial growth during or following food manipulation.

A major limitation of our study is that the food item identified by the case–control study, pasta salad with pesto, was no longer available for testing and thus there is no microbiological evidence that the pasta salad with pesto was contaminated with the outbreak strain. However, matrices with similar biochemical properties like potato salad have been confirmed before as vehicles of SFP in France [21] and Switzerland [15]. In the latter case, a strain with identical spa type t024 and enterotoxin A FRI100 allele led to the intoxication of 27 boy scouts. The *sea* gene found in our outbreak strain is the dominant *sea* allele described in *S. aureus* isolates that are associated with food poisoning outbreaks worldwide [19,21-23] and in enterotoxigenic isolates recovered from food handlers [24].

The epidemiological results from our carriage study are consistent with previous findings in similar studies. Our finding of 58% carriers among food handlers concurs with longitudinal studies showing that approximately 20% of persons are persistent nasal carriers and an additional 30% are intermittent carriers of *S. aureus* [25]. The high genetic diversity among asymptomatic carriers was also observed in similar studies in Germany [26], Switzerland [27], and Bosnia [28]. Interestingly, we found meticillin-susceptible livestock–associated strains with ST398 *spa* type t571 and variants thereof in both catering employees and in food. Similar clones have recently emerged causing severe infections in neighbouring France and Belgium [29,30], while remaining rare in Germany [31].

Although WGS has been applied to meticillin-resistant *S. aureus* in hospital and long-term care settings [32-34] and to other foodborne pathogens [35,36], to our knowledge our study is the first to report WGS as a tool in a staphylococcal food poisoning outbreak. While WGS showed virtually identical groupings to MLVA, one major advantage of WGS is that it is a universal method applicable to any bacterial species and that it provides further data on the presence of genes encoding virulence and resistance factors.

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Conflict of interest

None declared.

Authors' contributions

JM coordinated the various investigations, collated strains from different sources, constructed phylogenies, conducted the statistical analysis for the case–control study, and wrote the manuscript; FD conducted the classical genotyping including MLVA, spa typing and virulence factor detection by PCR; GM was responsible for the laboratory analysis of food items; CR and CO conducted the whole genome sequencing; CO assisted with bioinformatics and with preparing the figure; SJ provided reference material and assisted with interpretation; MP was responsible for the microbiological analysis of human strains; PH led the food inspection; PW was responsible for the public health response and the case–control data collection.

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6.5 Publication 17

Tracing and growth inhibition of *Staphylococcus aureus* in barbecue cheese production after product recall

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Tracing and inhibiting growth of *Staphylococcus aureus* in barbecue cheese production after product recall

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ABSTRACT

Staphylococcal food poisoning is one of the most prevalent causes of foodborne intoxication worldwide. It is caused by ingestion of enterotoxins formed by *Staphylococcus aureus* during growth in the food matrix. Following a recall of barbecue cheese due to the detection of staphylococcal enterotoxins in Switzerland in July 2015, we analyzed the production process of the respective dairy. Although most cheese-making processes involve acidification to inhibit the growth of pathogenic bacteria, barbecue cheese has to maintain a pH >6.0 to prevent undesired melting of the cheese. In addition, the dairy decided to retain the traditional manual production process of the barbecue cheese. In this study, therefore, we aimed to (1) trace *Staph. aureus* along the barbecue cheese production process, and (2) develop a sustainable strategy to inhibit growth of *Staph. aureus* and decrease the risk of staphylococcal food poisoning without changing the traditional production process. To this end, we traced *Staph. aureus* in a step-wise blinded process analysis on 4 different production days using *spa* (*Staphylococcus* protein A gene) typing, DNA microarray profiling, and pulsed-field gel electrophoresis analysis. We subsequently selected a new starter culture and used a model cheese production including a challenge test assay to assess its antagonistic effect on *Staph. aureus* growth, as well as its sensory and technological implications. We detected *Staph. aureus* in 30% (37/124) of the collected samples taken from the barbecue cheese production at the dairy. This included detection of *Staph. aureus* in the final product on all 4 production days, either after enrichment or using quantitative detection. We traced 2 enterotoxigenic *Staph. aureus* strains (t073/CC45 and t282/CC45) colonizing the nasal cavity and the forearms of the cheesemakers to the final product. In the challenge test assay, we were able to show that the new starter culture inhibited growth of *Staph. aureus* while meeting the sensory and technological requirements of barbecue cheese production.
Key words: *Staphylococcus aureus*, contamination routes, process analysis, barbecue cheese, starter culture

INTRODUCTION

In July 2015, the Swiss Federal Food Safety and Veterinary Office (Bern) issued a warning concerning the consumption of barbecue cheese, in which *Staphylococcus aureus* and staphylococcal enterotoxins (**SE**) had been detected. The dairy producing the barbecue cheese issued a recall, followed by a comprehensive external process analysis to trace *Staph. aureus* in the cheese-making process.

Staphylococcus aureus can cause staphylococcal food poisoning (**SFP**), the most prevalent foodborne intoxication worldwide. Ingestion of major or newly described SE (Hennekinne et al., 2010; Johler et al., 2015) formed during growth of the organism in food leads to symptoms of acute gastroenteritis and violent emesis (Hu and Nakane, 2014). Although symptoms usually subside within 24 h, SFP can, in rare cases, be fatal for children and the elderly. The Centers for Disease Control and Prevention (Atlanta, GA) estimates 240,000 cases per year in the United States, resulting in 1,000 hospitalizations and 6 deaths (Scallan et al., 2011).

As SE are heat-stable and will not be inactivated during the cooking process, preventive measures focus on inhibiting growth of *Staph. aureus* in the food matrix (Le Loir et al., 2003). To this end, starter cultures are used in the production of a wide range of foods including cheese. A suitable starter culture will outcompete the organism, thus effectively preventing *Staph. aureus* growth and SE formation. However, starter cultures need to meet several criteria to be suitable for the production of barbecue cheese. Although the antagonistic effect of many starter cultures is due to acidification of the food matrix, the pH of barbecue cheese cannot be lowered to values <6.0, as this would result in melting of the cheese when it is exposed to high temperatures during preparation by the customer. In addition, suitable starter cultures must have no negative sensory implications.

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In this study, we aimed to (1) trace *Staph. aureus* in the barbecue cheese production process, and (2) develop a sustainable strategy to inhibit growth of *Staph. aureus* and decrease the risk of SFP without changing the traditional production process.

MATERIALS AND METHODS

Sampling Along the Production Process and Isolation of Coagulase-Positive Staphylococci

To identify potential sources for contamination of the barbecue cheese at the dairy, 4 production cycles (see Figure 1) of the barbecue cheese were screened for coagulase-positive staphylococci in a blinded setup. To avoid bias, the dairy and the cheesemakers were only informed of the results upon completion of the study. Samples were taken from cheesemakers and at each step of the cheese-making process on August 24 ($\mathbf{T_1}$), August 27 ($\mathbf{T_2}$), September 1 ($\mathbf{T_3}$), and September 3 ($\mathbf{T_4}$), 2015 (see Table 1). Before the start of cheese production, swabs from the anterior nares and forearms of the cheesemakers were taken, as well as a swab from the inner and outer side of the end of the milk hose after pasteurization. All swabs were moistened using 0.85% NaCl. During cheese production, 10-mL samples of milk, whey, and starter cultures, and 30-g samples of curd and cheese were taken. Samples were screened for coagulase-positive staphylococci (**CPS**) using enrichment in Mueller-Hinton broth with 6.5% NaCl and plating on rabbit plasma fibrinogen (RPF) agar (Oxoid, Pratteln, Switzerland) and quantitatively, following the EN ISO 6888-2 protocol (ISO, 1999). Species identification of *Staph. aureus* was achieved through subsequent *spa* typing and the detection of species-specific markers by Staphytype DNA microarray profiling (Alere, Jena, Germany).

Cell Lysis and DNA Extraction

For cell lysis and DNA extraction, reagents of the Staphytype genotyping kit 2.0 and the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) were used according to the manufacturers' instructions. The concentration of nucleic acids was measured using a Nanodrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

spa Typing

The polymorphic X region of *spa* was amplified as previously described (Wattinger et al., 2012). Each PCR product was subsequently purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, Buchs, Switzerland) and sequencing was outsourced (Microsynth, Balgach, Switzerland). Subsequently, *spa* types were determined using the *spa* server (http://spa.ridom.de/; Harmsen et al., 2003).

DNA Microarray-Based Genotyping

The Staphytype genotyping kit 2.0 was used to detect the presence or absence of over 300 virulence and resistance genes and their allelic variants in *Staph. aureus* strains that were traced from the cheesemakers to the final product. Detection included genes encoding the major SE (*sea*, *seb*, *sec*, *sed*, *see*), as well as genes coding for newly described SE and enterotoxin-like superantigens (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *selm*, *seln*, *selo*, *seq*, *ser*, *selu*). Microarray profiles also allowed for assignment of the strains to *agr* types, as well as clonal complexes (**CC**; Monecke et al., 2008).

Pulsed-Field Gel Electrophoresis Analysis

Preparation of chromosomal DNA and pulsed-field gel electrophoresis (**PFGE**) analysis of *Sma*I-digested fragments was performed as previously described (Bannerman et al., 1995). Electrophoresis was carried out in a Bio-Rad CHEF-DR III electrophoresis cell (Bio-Rad, Hercules, CA). *Salmonella enterica* serovar Braenderup strain H9812 digested with 50 U of *Xba*I (12 h, 37°C) was used as a molecular size standard. Gels were analyzed with Gel Compar II software (Applied Maths, Sint-Martens-Latem, Belgium) using the dice coefficient and were represented by unweighted pair grouping by mathematical averaging (UPGMA) with an optimization of 0.5% and position tolerance of 1%.

Model Cheese Production Including Challenge Test Assay Using New Starter Culture

A new starter culture, consisting of CNS, was chosen (START Crudo 500, Christl Gewürze GmbH, Moosdorf, Austria). Species identification of CNS was performed by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS). To evaluate the suitability of the new starter culture for the barbecue cheese production, the cheesemakers produced 4 batches of model cheese (batches A to D) in a laboratory setting.

To assess growth of the starter culture during the cheese-making process, we determined CNS counts in 2 barbecue cheese production batches (batches A and B) at 3 time points. The first sample was taken from the curd directly after coagulation, the second sample was obtained from the cheese after pressing (same day), and the third sample was taken in the morning of the

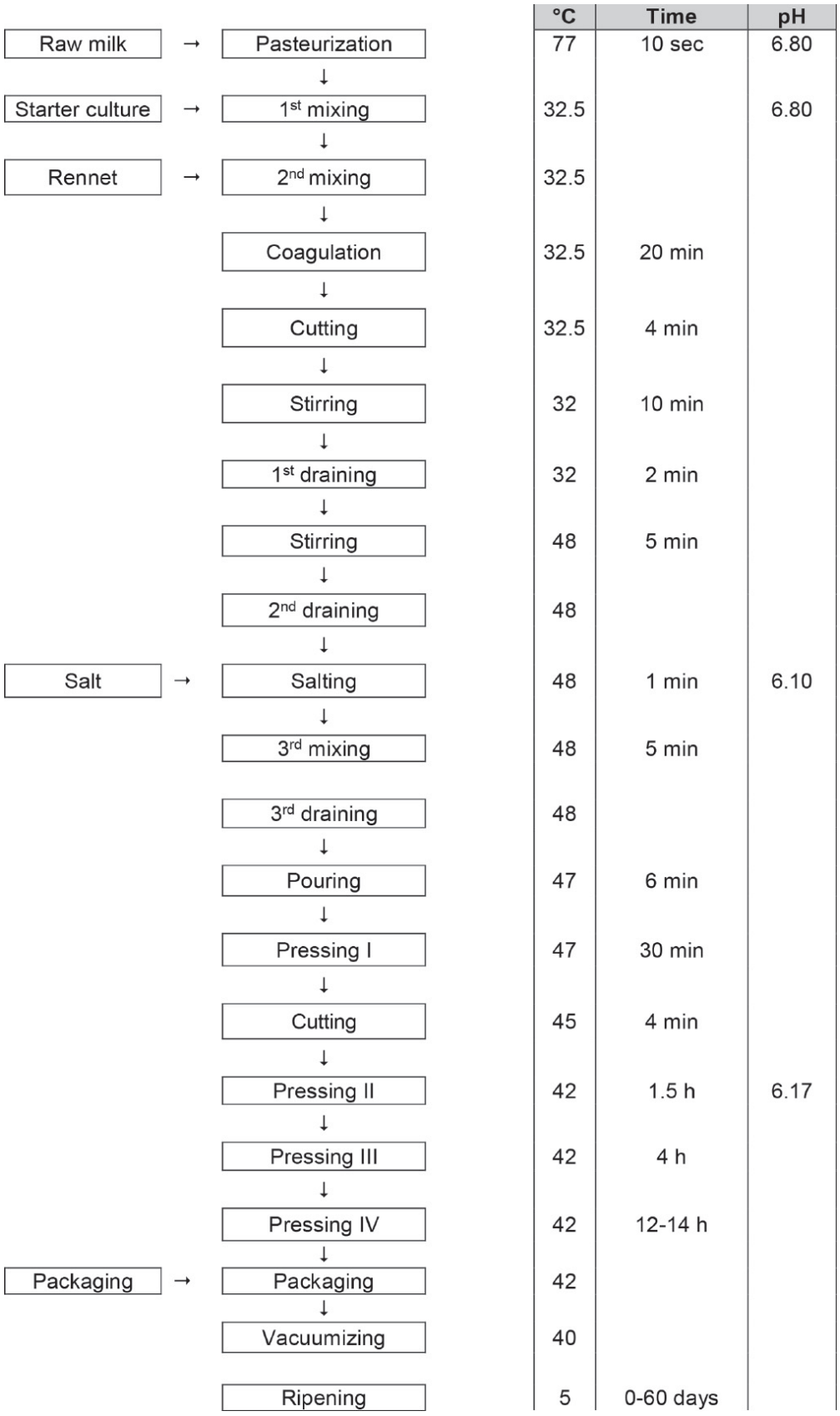

Figure 1. Flowchart providing an overview of the barbecue cheese production process until the ripening stage. The average batch size equaled 500 kg of milk, resulting in 55 kg of cheese.

following day. At this time, we also measured the pH of the cheese.

The inhibitory effect of the new starter culture on *Staph. aureus* growth was assessed in a challenge assay using 2 batches of barbecue cheese (batches C and D) produced with milk contaminated with *Staph. aureus*. To this end, a *Staph. aureus* strain isolated during the step-wise analysis of the barbecue cheese production process (strain 1, isolated at processing step P30, t282) was grown in brain-heart infusion (BHI) broth (Oxoid, Pratteln, Switzerland) at 37°C (225 rpm shaking) overnight to 2.5×10^9 cfu/mL. From the overnight broth, as well as from its 10-fold dilution in 0.85% NaCl, 1.0 mL was added to 3 L of milk, resulting in contamination levels of 10^6 cfu/mL milk in batch C and 10^5 cfu/mL milk in batch D. The cheeses were sampled after 24 h and CPS counts were determined following the EN ISO 6888-2 protocol (ISO, 1999).

RESULTS AND DISCUSSION

We detected *Staph. aureus* in 30% (37/124) of the samples collected along the production process of the 4 barbecue cheese production cycles at the dairy (see Table 1). This included detection of *Staph. aureus* in the final product on all 4 production days, either after enrichment (T_1) or by quantitative detection methods (T_2 to T_4). The highest CPS count detected in a sample equaled 6.3×10^5 cfu/g in the final product, with cell density levels of 10^5 to 10^6 cfu/mL generally

Table 1. Tracing *Staphylococcus aureus* in a step-wise blinded process analysis on 4 different days (T_1 to T_4)[1]

			Result[2]			
Sample ID	Source	Sampling details	T_1	T_2	T_3	T_4
Before processing (swabs)						
BP1	Cheesemaker 1	Nose	t282 (B)	—	t282 (B)	t282 (B)
BP2	Cheesemaker 2	Nose	t073 (A)	t073 (A)	t073 (A)	t073 (A)
BP3	Cheesemaker 1	Forearms	t282	$t362_e$	—	—
BP4	Cheesemaker 2	Forearms	—	t073 (A)	—	t5264
BP5	Milk hose after pasteurization	Inside and outside	—	—	—	—
Processing						
P1	Raw milk		t529	t3992	—	—
P2	Milk after pasteurization	Production start	—	—	—	—
P3	Milk after pasteurization	Mid-production	—	—	—	—
P4	Starter culture		—	—	—	—
P5	Curd before cutting	Sample 1	—	—	—	—
P6	Curd before cutting	Sample 2	—	—	—	—
P7	Curd before cutting	Sample 3	—	—	$t073_e$ (A)	—
P8	Curd after 1st stirring	Sample 1	—	$t073_q$ (A)	—	$t073_e$ (A)
P9	Curd after 1st stirring	Sample 2	—	—	—	—
P10	Curd after 1st stirring	Sample 3	—	$t073_e$	—	—
P11	Whey	1st draining	—	—	—	—
P12	Curd after 2nd stirring	Sample 1	—	—	—	$t282_e$ (B)
P13	Curd after 2nd stirring	Sample 2	—	—	—	$t073_e$ (A)
P14	Curd after 2nd stirring	Sample 3	—	—	—	—
P15	Whey	2nd draining	—	—	—	—
P16	Curd after mixing	Sample 1	—	—	—	—
P17	Curd after mixing	Sample 2	—	—	—	—
P18	Curd after mixing	Sample 3	—	—	—	—
P19	Whey	3rd draining	—	—	—	$t282_e$ (B)
P20	Cheese after pressing I	Sample 1	—	—	—	—
P21	Cheese after pressing I	Sample 2	—	—	—	$t282_e$
P22	Cheese after pressing I	Sample 3	—	—	—	$t282_e$
P23	Cheese after pressing II	Sample 1	—	—	—	$t5264_q$
P24	Cheese after pressing II	Sample 2	—	—	—	$t282_e$
P25	Cheese after pressing II	Sample 3	—	—	—	$t073_e$
P26	Cheese after pressing III	Sample 1	—	—	—	$t282_q$
P27	Cheese after pressing III	Sample 2	—	—	—	$t5264_q$
P28	Cheese after pressing III	Sample 3	—	—	—	$t282_q$
P29	Cheese after pressing IV	Sample 1	—	$t073_q$ (A)	—	$t5264_q$
P30	Cheese after pressing IV	Sample 2	—	$t073_q$	$t282_q$ (B)	$t282_q$
P31	Cheese after pressing IV	Sample 3	$t282_e$	$t073_q$	$t073_q$ (A)	$t282_q$

[1]The table indicates at which production steps *Staph. aureus* isolates were detected and provides an overview of the respective *spa* types (t073, t282, t5264, t529, t3992) and pulsed-field gel electrophoresis (PFGE) patterns (A, B) determined for selected isolates. Three strains could be traced from the cheesemakers to the final product: t072 (A), t282 (B), and t5264.

[2]Letters (A, B) in parentheses indicate assignment of isolates to PFGE patterns. Subscript q ($_q$) indicates that quantitative detection was possible for coagulase-postive staphylococci counts $>10^2$ cfu/g; subscript e ($_e$) indicates that lower levels were detectable only after enrichment.

Table 2. Molecular characteristics of the 3 *Staphylococcus aureus* strains traced from the cheesemakers to the final product

Characteristic	Strain 1	Strain 2	Strain 3
Typing			
spa typing	t282	t073	t5264
PFGE[1]	Pattern B	Pattern A	Not determined
Clonal complex	CC45	CC45	CC88
agr typing	*agr*I	*agr*I	*agr*III
Enterotoxin genes			
Major enterotoxins[2]	—	*sec*	—
Others[3]	*seg*, *sei*, *selm*, *seln*, *selo*, *selu*	*seg*, *sei*, *sel*, *selm*, *seln*, *selo*, *selu*	—

[1]Pulsed-field gel electrophoresis.

[2]Screening included all major enterotoxin genes (*sea*, *seb*, *sec*, *sed*, and *see*).

[3]Screening included *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *selm*, *seln*, *selo*, *seq*, *ser*, and *selu*.

being regarded as sufficient for enterotoxin production. The step-wise analysis showed that, in most cases, low-level contamination occurred early in the production process, with *Staph. aureus* being detected only after enrichment.

To determine the source of the contamination, we traced the isolates along the production process using *spa* typing, DNA microarray analysis, and PFGE analysis. The 37 isolates represented 6 different *spa* types: t282 (n = 15), t073 (n = 15), t5264 (n = 4), t362 (n = 1), t529 (n = 1), and t3992 (n = 1). The 3 *spa* types that were detected only once were found in *Staph. aureus* strains obtained from raw milk and the forearm of a cheesemaker. As these strains were not present in the final product, we excluded them from further characterization experiments. The DNA microarray analysis and *spa* typing showed that the remaining 34 isolates could be assigned to 3 distinct *Staph. aureus* strains. Genotypic characteristics, including typing results, as well as enterotoxin gene profiles of these strains are provided in Table 2. Strains 1 (t282/CC45) and 2 (t073/CC45) harbored enterotoxin genes and were repeatedly detected in samples from cheesemakers and in the final product. Strain identity of selected isolates was confirmed by PFGE analysis, resulting in only 2 distinct patterns. A third *Staph. aureus* strain (t5264/CC88) was only found in one production cycle (T_4) and was shown not to be enterotoxigenic.

Staphylococcus aureus colonizes the skin and mucosa of humans, with nasal carriage rates between 30 and 50% in the adult population (Diederen et al., 2006; Munckhof et al., 2008; Halablab et al., 2010; Wattinger et al., 2012). The 3 *Staph. aureus* strains detected in the nasal cavity and on the forearms of the cheesemakers, as well as in the final product, were assigned to t282/CC45, t073/CC45, and t5264/CC88. *Staphylococcus aureus* isolates of these *spa* types and clonal complexes have been previously reported in association with asymptomatic colonization and cases of infections in humans (Bloemendaal et al., 2009; Luedicke et al., 2010; Wattinger et al., 2012; Gómez-Sanz et al., 2013). Nasal carriage status of *Staph. aureus* is not necessarily permanent but has been shown to change frequently over time (Sakwinska et al., 2009). Although decolonization strategies such as intranasal application of mupirocin and chlorhexidine washing have high success rates immediately after treatment, many carriers will become recolonized during a longer follow-up period (van Rijen et al., 2008; Ammerlaan et al., 2009). Thus, decolonization of cheesemakers does not represent a sustainable approach to increase food safety and promotes the risk of nasal colonization with mupirocin-resistant *Staph. aureus* (Ammerlaan et al., 2009).

As the dairy decided to retain the traditional manual production of the barbecue cheese, the most promising strategy to increase food safety and reduce the risk of SFP was the inhibition of *Staph. aureus* growth by use of a new starter culture. The candidate starter culture was identified as *Staphylococcus vitulinus* by MALDI-TOF MS. As shown in Table 3, the new starter culture was able to grow under the conditions of the cheese-making process. It had no negative sensory implications and resulted in a favorable pH of the product (pH = 6.29). In the challenge assay, we observed that the starter culture was able to outcompete *Staph. aureus* (Table 4), with a maximum increase of CPS counts of 1 $\log_{10}$. This is particularly remarkable, as physical concentration effects alone can result in an increase of

Table 3. Growth of the starter culture evaluated by CNS counts using a model cheese production[1]

	Batch A		Batch B (+ *Staphylococcus aureus*)
Time point	CNS (cfu/g)	pH	CNS (cfu/g)
After coagulation	1.4×10^6	—	1.3×10^6
After pressing	2.2×10^5	—	2.5×10^5
Next morning	2.9×10^7	6.29	6.0×10^7

[1]Growth in presence of *Staph. aureus* was determined using an initial milk contamination level of 10^3 cfu *Staph. aureus* per milliliter of milk.

Table 4. Challenge test assay assessing inhibition of *Staphylococcus aureus* growth by the new starter culture in a model cheese production[1]

	Coagulase-positive staphylococci (CPS)		CNS
Batch	Artificial contamination in milk (cfu/mL)	After 24 h (cfu/g)	After 24 h (cfu/g)
C	10^6	2.0×10^7	2.4×10^8
D	10^5	6.0×10^5	2.1×10^8

[1]Growth of *Staph. aureus* and the new starter culture was quantified using CPS and CNS counts, respectively.

1 $\log_{10}$ during the cheese-making process (Peng et al., 2013).

CONCLUSIONS

We conclude that although the pasteurization process of the raw milk used for barbecue cheese production was successful, the risk of SFP persists due to *Staph. aureus* contamination by colonized cheesemakers. As decolonization of food handlers cannot ensure long-term freedom from nasal or skin carriage, the most promising strategy to increase food safety in the traditional manual production process is the use of a suitable starter culture. We were able to show that the new *Staphylococcus vitulinus* starter culture inhibited growth of *Staph. aureus* while meeting the sensory and technological requirements of barbecue cheese production. The dairy has now successfully implemented the new starter culture in the production process of the barbecue cheese and resumed regular production.

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7 Summary & Discussion

When this habilitation thesis was devised, the most likely sources of staphylococcal food poisoning were still unclear, the expression and regulation of enterotoxins under stress conditions encountered in the food matrix was poorly understood, and data on outbreaks and product-related studies was scarce. In addition, the role of newly-described staphylococcal enterotoxins in outbreaks was controversially discussed.

In this habilitation, a diversified approach was chosen to advance the scientific body of knowledge with regard to these research gaps, thus contributing to the overarching goal of minimizing the risk of staphylococcal food poisoning. To this end, data on the genomic level (**section 4.1**), the level of enterotoxin expression (**section 4.2**), and the level of outbreaks and products (**section 4.3**) were generated.

Variability in *seb, sec*, and *sed* gene and promoter sequences

To first gain deeper understanding of *S. aureus* enterotoxins and to enable selection of a representative set of strains for studies on enterotoxin expression and regulation under stress conditions, the genetic variability of the major staphylococcal enterotoxin genes *seb, sec,* and *sed* was investigated. The enterotoxin genes *sea* and *see* were not included, as they are encoded by prophages that may be induced by the tested stress conditions.

In a collection of enterotoxigenic *S. aureus* strains from humans and various animal species, the variability of *seb*, *sec,* and *sed* genes and promoters was determined by PCR amplification and sequencing. Several novel variants of enterotoxin promoter and gene nucleotide sequences were detected. While the *seb* promoter and gene sequences exhibited a high degree of variability, the *sec* and *sed* promoter and gene were more conserved. This is consistent with a study by Sato'o et al. (29) reporting novel *seb* variants carried by different *S. aureus* pathogenicity islands. Interestingly, a truncated variant of *sed* was detected in all *sed* positive strains isolated from rabbit carcasses. The *sed* variant exhibited a deletion resulting in a premature stop codon and a truncated *sed* amino acid precursor. While the protein was expressed in the majority of the tested strains, the deletion may impair the functionality of the protein and recognition by various detection methods.

Over the course of this habilitation, our dataset on promoter and enterotoxin variability was continuously extended, taking into consideration new findings with regard to previously unavailable full genome sequence data of specific clonal lineages and novel *S. aureus* pathogenicity islands carrying enterotoxin genes. We also contributed full genome sequences of the two staphylococcal food poisoning strains KLT6 and RKI4. KLT6 (Publication 1) represents an overproducer of SEB that was assigned to CC12, a clonal complex for which no full genome sequencing data had previously been available. In RKI4 (Publication 2), a novel pathogenicity island

carrying *seb* was identified. Recently, a manuscript detailing the compiled data on sequence variability in enterotoxin genes and promoters was prepared and submitted to Toxins.

S. aureus genomic characterization

A collection of *S. aureus* strains from outbreaks was assembled in cooperation with various national and international partners. In addition to the outbreak strains, hundreds of *S. aureus* isolates from potential sources of staphylococcal food poisoning were collected, including isolates from humans (nasal colonization and cases of infection), carcasses (pigs, poultry, rabbits), milk (bovine, ovine, caprine), and ready-to-eat foods.

In order to identify the most likely sources of staphylococcal food poisoning, all strains collected were characterized by *spa* typing and DNA microarray profiling. Characterization results were subsequently used to generate similarity trees using the SplitsTree software. Our results showed that food handlers colonized by or infected with *S. aureus* are the most common source of staphylococcal food poisoning outbreak strains (Publications 4, 6). In contrast, *S. aureus* isolated from pig, poultry, and rabbit carcasses (Publications 3, 5, 7) as well as from bovine, ovine, and caprine milk (Publications 3, 8) seem to play only a minor role in staphylococcal food poisoning outbreaks. This is consistent with the finding that *S. aureus* isolated from ready-to-eat foods were highly similar to isolates obtained from humans infected or colonized with *S. aureus* and did not exhibit clonal complexes or *spa* types characteristic for *S. aureus* adapted to animal hosts (Publication 6).

We also determined the population structure and virulence and resistance gene profiles of *S. aureus* from different sources. Our findings for *S. aureus* isolated from bovine, ovine, and caprine milk suggest that *S. aureus* shows pronounced host adaptation to cattle and to small ruminants. While some common characteristics among *S. aureus* from caprine, ovine, and bovine milk samples were observed, *S. aureus* from small ruminants form a distinct population differing from *S. aureus* adapted to the bovine udder (Publications 3, 8). In *S. aureus* isolated from pig carcasses, no genes encoding major enterotoxins and only very few other virulence genes were detected (Publication 3).

In our studies, we found *S. aureus* nasal carriage rates of 38% among asymptomatic adults (Publication 4), consistent with previous reports on nasal colonization (79). The single MRSA isolate detected among the asymptomatic nasal carriers in our study originated from a veterinarian working in an equine practice. The strain belonged to CC398 and *spa* type t011, which were also detected in Finland among clinical MRSA isolates from human patients and several horses (80) as well as from a fattening pig at slaughter (81). A recent study from Germany suggests that MRSA form equine infections nasally colonize 20% of veterinarians with occupational exposure to horses, but only very rarely cause infections in humans (82).

Based on the characterization data generated by sequencing and DNA microarray analysis,

suitable strains were selected for studies on enterotoxin expression and its regulation under stress conditions.

Enterotoxin expression and regulation under food-related stress conditions

Enterotoxin expression was quantified in different growth phases and under several food-related stress conditions in multiple wild type and isogenic regulatory knockout strains using a quantitative Real-Time approach and an ELISA assay.

In a first step, we identified suitable reference genes for the normalization of quantitative Real-Time PCR data under all tested stress conditions (Publication 9). Our data showed that even mild stress conditions significantly alter the expression of most housekeeping genes. This finding emphasizes the importance of validating the expression stability of reference gene candidates for quantitative Real Time PCR assays under stress conditions. The reference genes determined in this study cannot only be used for quantification of enterotoxin expression, but also for normalization of mRNA levels of any other gene under mild NaCl, glucose, nitrite, and lactic acid stress.

We were able to show that 4.5% NaCl (a_w = 0.97) and 30% glucose (a_w = 0.96) reduce enterotoxin D expression, whereas lactic acid stress (pH 6.0, a_w = 0.99) had no significant effect (Publications 10, 11). In contrast, nitrite stress (150 mg/l, a_w = 0.98) induced enterotoxin D expression in some strains (Publication 12). Further findings demonstrate that the production of staphylococcal enterotoxins cannot be reliably predicted based on viable cell counts. Therefore, legislative guidelines focused exclusively on viable cell counts need to be adjusted in order to assure consumer safety.

As for the effect of regulatory elements, we generated data on the effect of sigma factor B, SarA, and Agr on enterotoxin expression both under control and stress conditions. Under non-stress control conditions, all three regulators did not significantly alter *sed* mRNA levels. Under NaCl stress, loss of SarA resulted in a strain-specific increase or decrease of *sed* mRNA levels, and loss of SigB decreased *sed* mRNA levels. Neither on the transcriptional nor on the translational level, statistically significant effects of loss of Agr on enterotoxin D expression under control or stress conditions were observed. Previous studies suggest that Agr represents the main positive regulator of several staphylococcal enterotoxin genes, including *sed* (83–85). Interestingly, based on our findings, the role of Agr in the control of enterotoxin expression may have been overestimated in the past. Previous studies reporting decreased SEB, SEC, and SED production due to loss of Agr (83–85) used only a single strain ISP546 (86). ISP546 is a derivative of NCTC8325, a strain harboring a 11 base deletion in *rsbU*, an indirect positive regulator of sigma B (63, 87). As a defective sigma B operon was shown to affect global regulators Agr, Sar, and Rot, the results of regulatory studies based on NCTC8325 derivatives are most likely not representative (64–67). On protein level, loss of SigB increased and loss of SarA decreased *sed* expression. A similar effect of

these regulators on *seb* and *sec* expression was previously demonstrated (70, 88–90).

In general, the strong strain-specific variation detected in the expression of enterotoxins under stress conditions as well as the regulatory mechanisms controlling expression further emphasize the need for a multiple strain approach when investigating *S. aureus* virulence factor expression and regulation. Extensive further studies, using multiple well-characterized strains, will be needed to elucidate the complex intertwined network of regulators controlling the expression of staphylococcal enterotoxins and other virulence factors.

Outbreak investigations and product-related studies

To yield data on staphylococcal outbreaks and to determine effective strategies to minimize the risk of staphylococcal food poisoning, outbreak investigations were conducted and a cheese production process was optimized in order to inhibit *S. aureus* growth and enterotoxin production.

Investigating an outbreak that occurred at a wedding (Publication 13), we were able to link a *S. aureus* isolate detected in the feces of a patient to isolates of the same strain obtained from food and from the nasal swabs of a caterer. Outbreak investigations linking patient samples to food and to the original source of contamination are extremely rare (25). This was also the first study using not only PFGE and *spa* typing, but also Fourier-transform infrared spectroscopy (FTIR) for discrimination of *S. aureus* isolates in an outbreak context. The study thus represented a first stepping stone for our later work on the use of FTIR as a tool for high-resolution subtyping of *S. aureus* (91).

In another study investigating an outbreak among children and staff at a Swiss boarding school (Publication 14), we identified for the first time a *S. aureus* strain of genotype B in association with intoxications. Genotype B strains have been exclusively associated with very high (up to 65%) within-herd prevalence of mastitis (92). It is likely that the respective strain was present in high levels in the pooled milk of the affected herd and that growth and concentration effects during the production process of the raw milk cheese (93) further elevated these levels. In view of this outbreak and the possible occurrence of other foodborne pathogens in milk, consumers should be aware that consumption of raw milk and soft cheese produced from raw milk constitutes a health risk, in particular when children are involved. Interestingly, the data from this outbreak investigation also suggest that incubation periods can depend of the age of the patient, with 2.5 h in children under 10 years of age, 3.5 h in older children and teenagers, and 7 h in adults.

The role of newly-described enterotoxins in staphylococcal food poisoning outbreaks had been controversially discussed for years, although most of these toxins elicit an emetic response in the monkey feeding assay (21, 35). In many outbreaks, strains exhibiting both major and newly described enterotoxin genes can be detected, but only major enterotoxins are identified in food and

feces, as only for these toxins immunological detection methods are commercially available. Therefore, even if only low levels of SEA-SEE will be detected in food or feces, the outbreak will be attributed to one of the major enterotoxins. And even if food and feces samples should yield negative results for major enterotoxins and strains harboring *seg, sei, sem, sen,* or *seo* were found, the outbreak may not be reported as investigators question the relevance of these newly-described enterotoxins. Prompted by an outbreak that occurred in September 2014 in the Ticino region, in which no classical enterotoxins were detected, we screened the records of the Swiss Federal Office of Public Health for evidence of staphylococcal food poisoning outbreaks potentially caused by *S. aureus* producing newly-described enterotoxins only. Using epidemiological data from two different outbreaks, we were able to provide further evidence that newly-described staphylococcal enterotoxins encoded by the *egc* cluster can cause outbreaks of staphylococcal food poisoning (Publication 15).

In an investigation of an outbreak that occurred at an equine sports event in Luxembourg, we employed for the first time whole genome sequencing as a tool in a staphylococcal food poisoning outbreak investigation, complemented by carriage and case-control studies (Publication 16).

We also adapted a production process to minimize the risk of staphylococcal food poisoning. Following a recall of barbecue cheese, we traced *S. aureus* in a step-wise blinded process analysis in a dairy. We were able to trace two enterotoxigenic *S. aureus* strains colonizing the nares and forearms of the cheesemakers to the final product. As decolonization of food handlers cannot ensure long-term freedom from carriage (6, 94), the most promising strategy to increase food safety in the traditional manual production process was the use of a suitable starter culture. However, the selection of a suitable starter culture for barbecue cheese production is particularly challenging not only for sensory, but also for technological reasons. The antagonistic effects of most starter cultures are due to acidification. However, barbecue cheese has to maintain a pH >6.0 to prevent undesired melting of the cheese during food preparation by the customer. A new starter culture was selected and identified as *Staphylococcus vitulinus*. The starter culture was subsequently tested in a model cheese production and a challenge assay to evaluate its antagonistic effect on *S. aureus*. We were able to show that the new starter culture met all sensory and technological requirements, and inhibited growth of *S. aureus*, thus minimizing the risk of staphylococcal food poisoning.

8 Outlook

To gain further insights into enterotoxin D expression during food production and preservation, western blotting experiments quantifying SED production in different strains are being performed. As protein A could interfere with SED quantification by western blotting, the *spa* gene is currently being knocked out in several strains.

Another major follow-up project currently conducted is focused on extending the experiments on enterotoxin expression under food-related stress described in this habilitation, in order to include data on the expression of other enterotoxin genes such as *seb*. The sequencing data generated that we generated showed that the *seb* gene and promoter sequence are extremely variable. In addition, the gene can be located on different *S. aureus* pathogenicity islands and is present in only a single copy in the chromosome. As a quantitative Real-Time approach to measure *seb* expression is therefore unlikely to yield satisfactory results, we decided to use transcriptional fusions of different *seb* promoters and the reporter gene *blaZ*.

A SEB overproducer (KLT6), a SEB reference strain (S6C), and a SEB low-level producer (COL) were selected, based on SEB levels determined by western blotting. The different promoter sequences were PCR amplified and cloned into a pGem vector, before being cloned into pCN41 (95). As the resulting construct would integrate at the SaPI1/3 site into the chromosome, *seb* positive target strains would loose SaPI3 carrying *seb* and adjacent regions in the process. Therefore, the pCN construct was cloned into vectors pJC1648 (Cd) and pJC1649 (Erm), kindly provided by John Chen (Skirball Institute of Biomolecular Medicine, New York University), which insert into the chromosome at the SaPI4 site. The pJC construct was subsequently electroporated into the restriction-negative laboratory strain RN4220 and transduced into reference strains of known background USA300 and HG003 (96) using phage 80alpha.

Currently, a nitrocefin assay is performed, in which enzymatic activity of beta galactosidase is used to determine the activity of the different *seb* promoters under NaCl, nitrite, lactic acid, and glucose stress conditions in different genomic backgrounds (see cover illustration). Preliminary data indicates that *seb* promoter activity under food-related stress conditions is dependent on both the promoter sequence and the background of the strain.

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